

AD _____

Award Number: DAMD17-00-1-0449

TITLE: The Contribution of P27Kip1 Regulation to Tamoxifen
Resistance

PRINCIPAL INVESTIGATOR: Jeffrey C. Donovan
Doctor Joyce Slingerland

CONTRACTING ORGANIZATION: Sunnybrook and Women's College
Toronto, Ontario, Canada M4N 3M5

REPORT DATE: May 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20021104 007

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2002	3. REPORT TYPE AND DATES COVERED Annual Summary (1 May 01 - 30 Apr 02)	
4. TITLE AND SUBTITLE The Contribution of P27Kip1 Regulation to Tamoxifen Resistance			5. FUNDING NUMBERS DAMD17-00-1-0449	
6. AUTHOR(S) Jeffrey C. Donovan Doctor Joyce Slingerland				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Sunnybrook and Women's College Toronto, Ontario, Canada M4N 3M5 E-mail: jdonovan@srcl.sunnybrookutoronto.ca			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) In the course of this project, I investigated the role of the cell cycle inhibitor P27 in response to antiestrogens and TGF- β . Using ER positive MCF-7 lines, this work revealed P27 is an essential mediator of G1 arrest by antiestrogens. In resistant cell lines P27 deregulation was found to occur via increased MAPK activation. Inhibition of MAPK signaling restored antiestrogen sensitivity. P27 is not only an essential mediator of antiestrogens, but also plays a role in G1 arrest by TGF- β . P27 was an essential mediator of G1 arrest in malignant breast cancer lines, but not in normal finite lifespan mammary epithelial cells. Compensation by P21 and P130 allowed maintenance of G1 arrest despite forced P27 deregulation.				
14. SUBJECT TERMS P27, MAPK, tamoxifen resistance, P21, cyclin E, cdk2, breast cancer			15. NUMBER OF PAGES 130	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

TABLE OF CONTENTS

Cover.....	
SF 298.....	
Preface.....	3
Introduction (SECTION I)	5
Body.....	
SECTION II	29
SECTION III	45
SECTION IV	66
SECTION V	67
Key Research Accomplishments.....	85
Reportable Outcomes.....	87
Conclusions (SECTION VI)	89
References.....	95
Appendices.....	107

PREFACE: Final DOD Report Overview

This Final Report addresses the role of the cyclin dependent kinase inhibitor p27 in the cell cycle arrest by antiestrogens and TGF- β . The work is divided into six sections (I-VI).

Section I is the Introduction and reviews the basic mechanics of the cell cycle with emphasis on the G1 phase. This is followed by a discussion of mechanisms by which cell cycle regulators become altered in cancer, and the prognostic significance of such alterations. The chapter concludes with an overview of how antiestrogens and TGF- β cause cell cycle arrest and the mechanisms that lead to antiestrogen and TGF- β resistance.

Section II describes the changes in cell cycle profile and cell cycle proteins that occur following treatment of ER positive MCF-7 breast cancer cells with estrogens and antiestrogens. These studies demonstrated that both p21 and p27 are key effectors of G1 arrest by antiestrogens. These studies have been published in *Proceedings of the National Academy of Science* and are found in the Appendix. These studies were reported in the previous Annual Report to the US Army.

In Section III, antiestrogen resistant cell lines were studied to determine whether antiestrogen resistance was associated with altered p27. Only data from Figures 1 and 3 were complete at the time of last year's Annual Report to the Army. Evidence shown in Section III supports that increased active MAPK levels contributed to the antiestrogen resistance and altered p27 regulation.

In Section IV, I review work which is presently conducted by the applicant although will not be completed during the time in which the applicant will hold his award. We are immunostaining paraffin embedded, formalin fixed breast cancer tissues with both active MAPK antibodies and p27 antibodies to determine whether MAPK activation in breast cancer is associated with p27 loss and antiestrogen resistance.

In Section V, normal non-malignant and malignant breast cells were used to compare the effect of the antisense mediated inhibition of p27 expression on the maintenance of G1 arrest by TGF- β . These studies extend from our studies of the role of p27 in response to antiestrogens. These studies have only recently been completed, and we are in the process of submitting the manuscript. These data support a compensatory role for p21 and p130 in maintaining G1 cell cycle arrest in non-malignant, but not malignant cells following inhibition of p27 expression. Impaired checkpoint controls during malignant tumor progression may alter the role of p27 from an essential to redundant inhibitor of G1-to-S phase progression.

Finally, Section VI provides a summary of this Final Report.

Section I
INTRODUCTION

INTRODUCTION

Since this Research supported by the Department of the Army investigates mechanisms of cell cycle regulation by estrogens, antiestrogens and TGF- β , I will begin with a general overview of the cell cycle. The cell cycle is composed of four phases (see Fig. 1 and for review see¹).

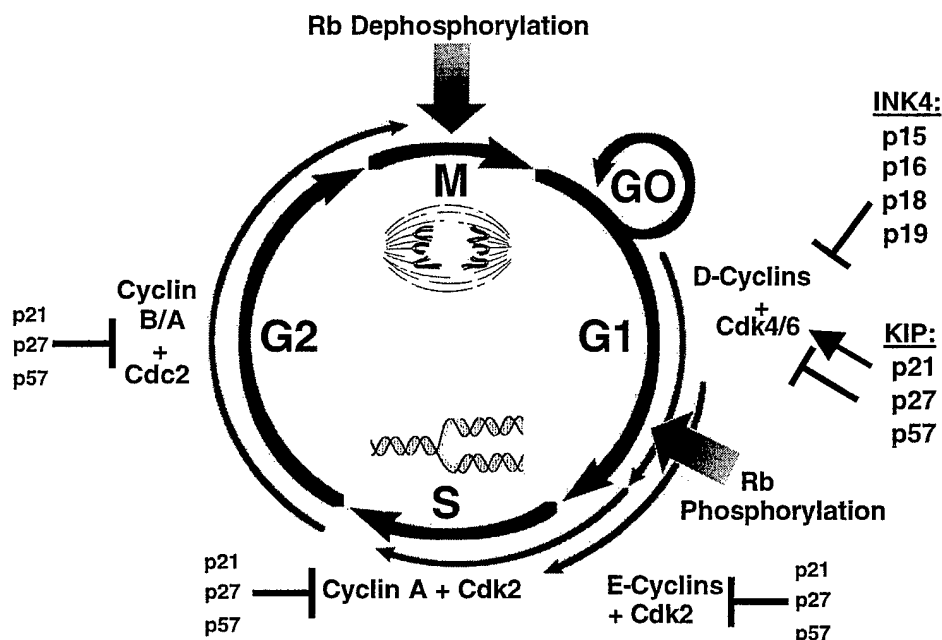


Figure 1. The cell cycle. The major mammalian cyclin-CDK complexes regulating the four cell cycle transit are shown, along with the CDK inhibitors involved in their kinase inhibition. While INK4 proteins act as inhibitors of CDK4 and CDK6, KIP family members are regarded as assemblers (activators) of cyclin D-CDK4 or cyclin D-CDK6 complexes and inhibitors of the remaining CDKs.

The gaps (G) between mitosis (M phase) and DNA replication in S phase are respectively called the G1 and G2 phases. The G1, S and G2 phases are collectively referred to as Interphase. Following mitosis, cells may exit the proliferation cycle and enter a quiescent G0 phase in the absence of appropriate stimuli triggering further cell division cycles. Cells in G0 or G1 are receptive to signals in the extracellular environment. In the presence of a sustained mitogenic stimulus, cells exit G0 and progress through the G1 phase to a final commitment stage known as the "restriction point,"

or R point (see Fig 1). The R point has been defined in cultured cells as the point in the G1 phase beyond which growth factors are no longer needed for completion of the S, G2, and M phases. Passage through the R point is one of the most precisely controlled events during the cell cycle. In cancer cells, the deregulation of various components of the G1 cell cycle machinery results in cells with different degrees of autonomy from extracellular growth stimulatory or growth inhibitory signals, in turn making them more likely to meet the requirements for transition through the R point. Thus, G1 cell cycle defects, which have been identified in at least 90 % of human cancers, often facilitate passage through the restriction point.

MECHANISMS OF CDK REGULATION

Progression through the cell cycle is catalyzed by a family of serine-threonine protein kinases (Cdk1-10). Cdk's are subject to regulation via multiple mechanisms, including site-specific phosphorylation, the binding of activator molecules known as cyclins, and the binding of Cdk inhibitors (for review see ^{2,3}).

Cdk regulation by phosphorylation

Phosphorylation of a conserved threonine residue, located in the catalytic cleft of the kinase (e.g. Thr 161 for Cdk1, Thr 160 for Cdk2) is required for full Cdk activation and this is catalyzed by the Cdk-activating kinase (CAK) ⁴ or the recently identified Cak1p ⁵. Inhibition of CAK action on, or CAK access to, the Cdk's prevents the activating Cdk phosphorylation and leads to cell cycle arrest.

Cdc25 phosphatase family members, Cdc25A, Cdc25B, and Cdc25C, remove the inhibitory phosphates from the Thr 14 and Tyr 15 sites on Cdk's and like CAK, are also required for full Cdk activation (Fig. 2). The expression and activity of these phosphatases is periodic depending on the phase of the cell cycle. Cdc25A expression and activity is maximal at the G1/S transition and

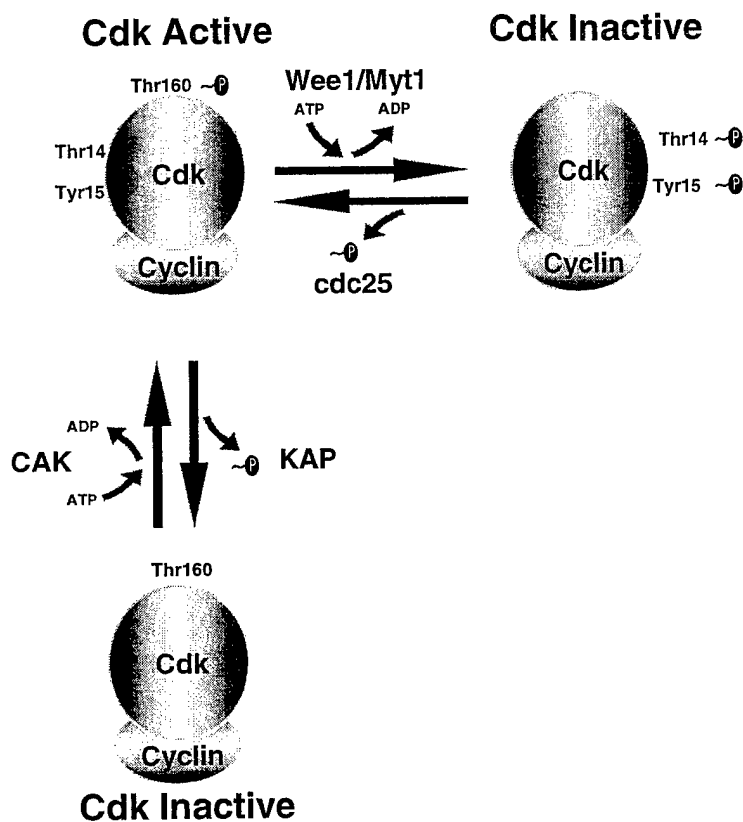


Figure 2. CDK regulation by phosphorylation. Phosphorylation of Thr 160 by the CAK and dephosphorylation of Thr 14/Tyr15 residues by Cdc25 family members is essential for CDK activation. Opposing these CDK activating events are the KAP phosphatase and the Wee1/Myt1 kinase, respectively.

contributes to the activation of Cdk2^{6,7}. In certain cell types, Cdc25A expression is induced by c-myc and Cdc25A may be activated by Cdk2 via a positive feedback loop^{6,8}. Overexpression of Cdc25A accelerates the G1/S transition, which suggests that this phosphatase controls a rate-limiting step needed for S phase entry⁹. Micro-injection of anti-Cdc25A antibodies arrest cells in G1. Cdc25B and Cdc25C are involved in the regulation of the G2/M transition and act on the cyclin B-associated Cdk1¹⁰. In addition to activating phosphorylation, Cdks are subject to inhibitory

phosphorylation. Phosphorylation at conserved inhibitory sites (Thr 14 and Tyr 15 on Cdks) leads to Cdk inhibition and this is catalyzed by the WEE-1 and Myt-1 kinases (Fig. 2).

Cdk activation by cyclin binding

The binding of a cyclin molecule is a requirement for Cdk activation *in vivo*¹¹. The cyclins include a family of proteins (cyclin A to H) that share a conserved sequence of about 100 amino acids, referred to as the cyclin box. Cyclin binding to the Cdk alters the conformation of the Cdk and contributes to its activation. Activated cyclin-Cdk complexes, in turn, phosphorylate various target proteins to ultimately mediate progression through the various cell cycle phases (Fig. 2). The precise timing of cyclin-Cdk activation during the cell cycle ensures that complexes are catalytically active only when they are needed^{12,13}. This is regulated by the specific subcellular localization and the timed expression and degradation of various cyclins and Cdk inhibitors throughout the cell cycle. Cyclins are also regulated at the level of transcription. In general, the peak nuclear expression of a specific cyclin occurs when the peak activity of the partner kinase is required, and following activation, the cyclins are rapidly degraded by ubiquitin-mediated proteolysis.

Progression through G1 and into S phase is regulated by the activities of the cyclin D-, cyclin E- and cyclin A-associated kinases^{2,14}. In most cells, cyclin D-Cdk complexes are activated by mitogenic stimuli early in G1 followed by activation of cyclin E-Cdk2 in mid-G1 (Fig. 1). The D and E-type cyclins are essential for movement through the G1/S transition: microinjection of antibodies to cyclin D1¹⁵, cyclin E1¹⁶, or cyclin E2¹⁷ can prevent G1-to-S phase progression. Overexpression of both the D-type cyclins and cyclin E can shorten the time needed to progress from G1 into S phase^{16,18,19}.

One of the main targets of G1 Cdks is the retinoblastoma protein, pRb (Fig. 1). In early G1, pRb is hypophosphorylated and bound to a member of the E2F family of transcription factors.

These pRb/E2F complexes recruit additional molecules such as histone deacetylases to repress the transcription of genes whose products are required for S phase entrance, such as cyclin E and cyclin A as well as enzymes needed for DNA synthesis. Cyclin D1-dependent and cyclin E-dependent Cdk's both contribute to pRb phosphorylation during G1-to-S phase. E2F family members dissociate from the hyperphosphorylated pRb, and on release, activate gene transcription. The phosphorylation of the retinoblastoma protein is one indicator of cell cycle progression through the restriction point.

Cyclin A-Cdk2 activation in late G1 follows cyclin E-Cdk activation and is essential for initiation of and progression through S phase and for the onset of mitosis²¹. Antibodies to cyclin A block S phase entry and ectopic expression accelerates S phase entry²². In humans, two B-type cyclins (cyclin B1 and cyclin B2) associate with Cdk1 (also known as cdc2) to regulate entry into and exit from mitosis (for review see²³). The Cdc25C phosphatase plays an important role at the G2/M transition by regulating cyclin B-Cdk1 activation and hence entry into mitosis²³.

The Cdk Inhibitors

In addition to phosphorylation and cyclin binding, Cdk's are also subject to regulation by the binding of Cdk inhibitory (CKI) proteins. There are two families of CKIs: the Kinase Inhibitory Protein (KIP) family and the Inhibitor of Cdk4 (INK4) family²⁴. The KIP family members, which include p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, share homology in the N terminal Cdk inhibitory domain, and in vitro they can inhibit all cyclin-Cdk complexes. In vivo, KIP expression and activity is tightly regulated. Overexpression of either KIP or INK proteins leads to G1 arrest. Only a single KIP molecule is required for cyclin/Cdk inhibition²⁵. The KIP protein p27 has been a focus of this work supported by the Department of the Army. p27 was identified in cells arrested in the G1 phase by the cytokine transforming growth factor beta (TGF- β)^{30,31}, by contact inhibition^{31,32} and by the drug lovostatin^{32,33,34}. Accumulation of p27 in cyclin E/Cdk2 complexes induces and/or maintains G1 arrest in response to several anti-proliferative signals. p27 plays an essential role in maintaining G1

arrest in serum starved fibroblasts³⁵, and as I demonstrate in Section II, also in antiestrogen treated breast cancer cells³⁶. The observation that p27 knockout mice are larger than p27 wild type mice and have multiorgan hyperplasia further support a role for p27 in proliferative control³⁷⁻³⁹. p27 is largely regulated by translational controls and by ubiquitin-mediated proteolysis. The increased cyclin E-Cdk2 activity in late G1 leads to phosphorylation of p27 at Thr 187^{40,41}, allowing for recognition of p27 by the F-box protein Skp2 leading ultimately to the ubiquitin-mediated proteolysis of p27 (Fig. 3A)^{42,43}.

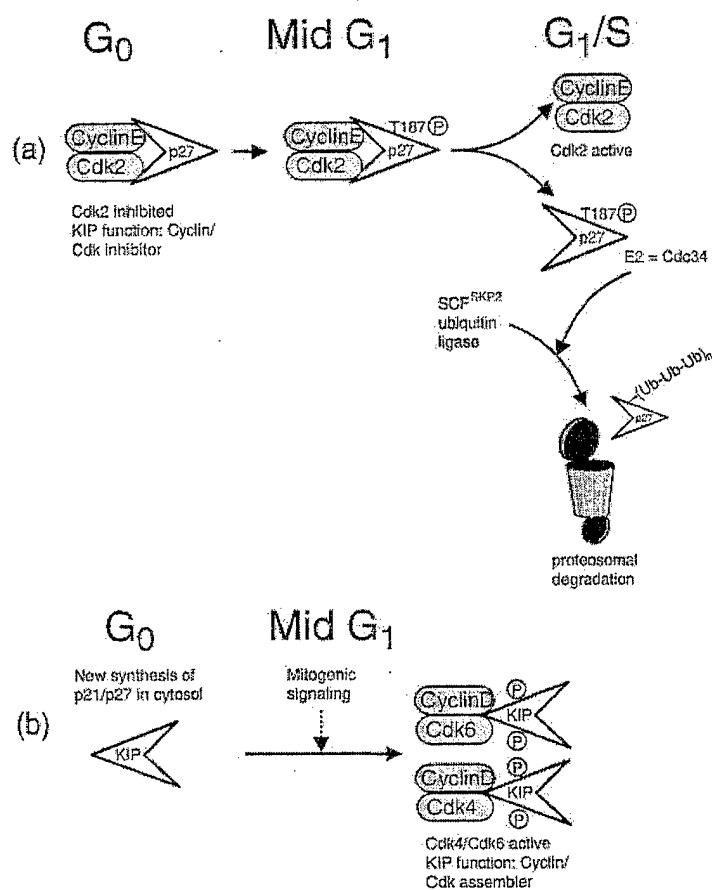


Figure 3. Dual function of KIP family members in G1. (a) KIP family members such as p27 act as cyclin E-CDK2 inhibitors in G₀. Phosphorylation of p27 on Thr 187 targets p27 for recognition by the F-box protein Skp2 which ultimately leads to ubiquitination and proteasomal degradation of p27 in late G₁. The reduction in KIP levels is required for cyclin E-CDK activation at the G₁/S transition. Skp2 independent p27 degradation may also occur in the early G₁ phase. (b) In addition to their role as CDK inhibitors, KIP proteins facilitate the assembly and activation of cyclin D-CKD4 or cyclin D-CKD6 complexes in mid-G₁.

Thus, p27 acts as an inhibitor of cyclin E-Cdk2 in the early G1, but as a Cdk2 substrate in late G1^{11,44}. Skp2 levels are low in quiescent cells (where p27 levels are high); growth factor stimulation leads to induction of Skp2. Skp2 overexpression in quiescent fibroblasts promotes cell cycle entry^{43,45}, and antibodies to Skp2 inhibit G1-to-S phase progression. In contrast to p27 degradation in late G1, p27 degradation in early-to mid G1 appears to be independent of T187 phosphorylation and Skp2. Skp2^{-/-} cells show early G1 proteolysis of p27 as do mutant T187A p27 murine knock-in cells. Thus, there may be at least two pathways for p27 degradation during G1-to-S phase progression.

Alterations in the KIP family members in cancer

As negative regulators of the cell cycle, the KIP and INK4 families of Cdk inhibitors, are frequently targets for inactivation in cancer (for review see⁸⁷). Mutations of the genes encoding the KIP family members are infrequent in human tumors, but alterations at the level of KIP gene expression or KIP protein stability are not uncommon. The prognostic value of p21 protein levels is controversial in human cancer. In breast cancer, for example, a few relatively small studies have demonstrated an association between low p21 levels and poor prognosis, whereas other studies have indicated that high p21 levels may predict poor outcome.

Of the Cdk inhibitors examined to date, p27 may have the greatest potential as a clinically relevant prognostic factor (for review see⁸⁸). Mutations or deletions in the p27 gene are rare in cancers. However, reduction in p27 protein levels has been observed in primary cancers of the breast, colon, lung, prostate, stomach and esophagus and its loss is associated with increased tumor grade. Moreover low p27 is an independent predictor of poor patient prognosis in a number of studies by multivariate analysis⁸⁸. The reduced p27 level in human tumors results from increased degradation of the protein via the ubiquitin-proteasome pathway⁸⁹. Extracts from several cancers with low p27 protein levels have demonstrated an elevated proteolytic capacity towards recombinant

p27. In some, but not all cases, the F-box protein Skp2 may contribute to the increased p27 degradation. In human lymphomas, oral cancers and colorectal cancers, increased Skp2 expression correlates positively with the grade of malignancy and inversely with p27 levels ⁹⁰⁻⁹².

In addition to reduction in KIP levels, the mislocalization of p27 within the cytoplasm was associated with poorer survival in Barrett's Associated Adenocarcinoma of the esophagus ⁹³. In 3 different studies of primary breast cancers, approximately 42 % of cancers were shown to have both nuclear and cytoplasmic p27 compared to only nuclear p27 in the normal breast tissue (Liang and Slingerland, unpublished, Viglietto, unpublished and Arteaga, unpublished). In one study, cytoplasmic p27 was associated with higher tumor grade, and both reduced disease free and overall survival (Liang and Slingerland, unpublished). These three studies demonstrated that oncogenic activation of the PKB pathway altered p27 phosphorylation causing its cytoplasmic mislocalization in cultured cells. Moreover, they showed a strong association between cytoplasmic p27 and elevated protein kinase B activity in primary breast cancers. Activation of the PKB pathway has also been shown to correlate with p21. In one study, this was associated with cytoplasmic mislocalization of p21 ⁹⁴, but in 2 other reports, PKB activation led to p21 phosphorylation but this was not associated with cytoplasmic mislocalization of the protein ^{95,96}. The overexpression of the HER-2 receptor tyrosine kinase, which is seen in up to 30% of breast cancers and associated with poor prognosis, has been implicated in the enhanced ubiquitin-mediated degradation of p27 and the cytoplasmic mislocalization of p27 through activation of the MAPK and PI3K/PKB pathways, respectively.

ONCOGENE ACTIVATION LEADS TO ALTERED PROLIFERATIVE AND ANTI-PROLIFERATIVE CONTROLS

Normal cell cycle regulation requires the function of the Ras protooncogene product. Altered Ras expression and/or activity are common in many cancers including breast cancer, and both contribute to altered regulation of G1 cell cycle control. Ras proteins are guanosine triphosphate

binding proteins that play a pivotal role in control of many cellular processes, including differentiation and proliferation. Ras activity is required for cell cycle progression⁵⁹. Overexpression of ras in serum starved NIH 3T3 triggers cells to enter S phase; conversely microinjection of anti-ras antibodies in the presence of complete serum, prevents S phase entry. A key effect of ras action in late G1 is the activation of pathways that contribute to phosphorylation of the retinoblastoma protein.

Mitogenic stimulation of quiescent cells from G0 into S phase involves a biphasic pattern of ras activation: an early G1 phase involving activation of the Ras-Raf-MEK-mitogen-activated – protein kinase pathway (MAPK) cascade and a mid-G1 phase involving activation of the ras-phosphatidyl-inositol-3-kinase (PI3K)-protein kinase B (PKB) pathway^{60,61}. Both of these pathways play important roles in the regulation of the cell cycle machinery (for detailed review see⁶²).

Multiple Ras effector pathways regulate cyclin D1. Activation of the ras-Raf-MEK-MAPK pathway activates cyclin D1 gene transcription and may regulate the assembly and activation of cyclin D1-Cdk4 complexes⁶³. The PI3K-PKB pathway plays an important role in the post-translational regulation of cyclin D1. PKB inhibits the glycogen synthetase-kinase-3-beta (GSK-3 β). The GSK-3 β mediated phosphorylation of cyclin D1 on Thr-286 regulates cyclin D1 nuclear export and cytoplasmic degradation^{64,65}. The PI3K/PKB pathway may also enhance cyclin D1 transcription and translation⁶⁶. Thus, PKB activation increases cyclin D1 levels.

Multiple ras effector pathways also regulate p27. Constitutive Ras-MEK-MAPK signaling has been shown to accelerate p27 proteolysis in some cell types, and inhibition of the pathway by pharmacological MEK inhibitors increased p27 levels and prevented S phase entry^{67,68}. There may be important differences in the contribution of MAPK signaling to p27 regulation in fibroblasts and epithelial cells. In some cell types, including NIH 3T3 cells, MEK activation did not reduce p27 protein levels, but rather facilitated p27 sequestration by cyclin D1-Cdk4 complexes⁶³. *In work of*

*this Report (Section III), I show that constitutive activation of MAPK can cause resistance of breast cancer cells to the antiestrogen tamoxifen, in part by altering p27's phosphorylation state, leading to a loss of its cyclin E1-Cdk2 inhibitory function*⁶⁹

CELL CYCLE REGULATION BY ESTROGENS AND ANTIESTROGENS

This Final Report addresses how estrogens and antiestrogens affect the cell cycle in estrogen receptor positive breast cancer cells. I also investigated how breast cancer cells become resistant to antiestrogens. Having provided a brief overview of the cell cycle, I will now provide a brief introductory review of how estrogens drive cell cycle progression in hormonally responsive breast cancer cells, and how these effects are opposed by antiestrogens.

Estrogen is a potent mitogen for many breast cancer cells, and stimulates quiescent G0 arrested ER positive breast cancer cells to enter the cell division cycle. The ability of estradiol to stimulate proliferation is dependent on the presence of a functional estrogen receptor (ER) (for review see¹⁰¹ and Figure 4). The two estrogen receptors, ER α and ER β are both expressed in breast cancer tissues.

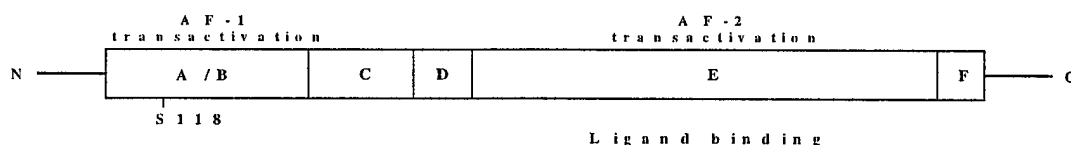


Figure 4. The estrogen receptor. The ER α contains several important domains. The carboxy terminal hormone-binding domain in region E contains an estrogen inducible transcriptional activating function known as AF-2. A second constitutively active transcriptional activating domain known as AF-1 is located in the A/B N-terminal region of the ER. Between the AF-1 and AF-2 regions are the DNA binding domain (C) and the hinge region (D). In the absence of estradiol, ER binds heat shock proteins in the cytoplasm^{102,103}. Estradiol binding to the ER leads to dissociation of heat shock proteins (Hsp70/90) and allows receptors to homodimerize, bind to additional regulatory proteins and then bind to an estrogen response element (ERE) in promoter region of target genes to induce transcription. More recently it has been demonstrated that the ER may also bind to other transcription factors such as Sp-1, AP-1 and NF-kB to induce gene transcription in a non-ERE dependent manner¹⁰¹.

Several groups have shown that estrogen stimulation of quiescent ER positive MCF-7 breast cancer cells leads to the rapid induction of cyclin D1 and accumulation of active cyclin D1-Cdk4-KIP complexes (See Fig 5). Upregulation of cyclin D1 plays an essential role during estradiol stimulated G1-to-S phase progression through effects on pRb phosphorylation. Microinjection of anti-cyclin D1 antibodies inhibits S phase entry in response to estradiol ¹⁰⁴. Estrogens also stimulate G1-to-S phase progression through reduction in steady state KIP binding to cyclin E-cdk2 and activation of this kinase. *In the studies to be discussed in Section II, I specifically tested whether the inhibition of KIP expression in ER-positive, estrogen-deprived cells using antisense oligonucleotides could mimic estrogen-induced cell cycle progression.*

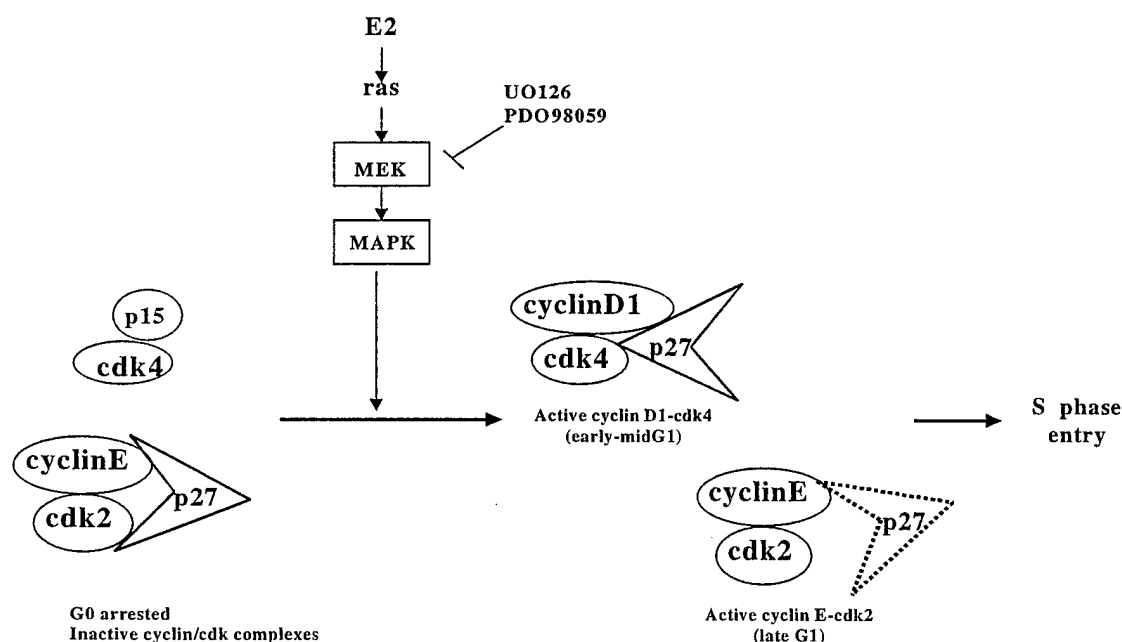


Figure 5. MAPK is an essential mediator of cell cycle progression. Estradiol-stimulated cell cycle entry leads to MAPK activation and the activation of cyclin D1-Cdk4 complexes in early-mid G1 followed by activation of cyclin E-Cdk2 complexes in late G1. p27 plays an important role in the assembly and activation of cyclin D1-Cdk4 complexes during G1-to-S phase progression and inhibits cyclin E-Cdk2 during cell cycle arrest. Inhibition of MEK activity with U0126 or PD098059 prevents the estradiol mediated activation of these cyclin/Cdk complexes and prevents progression of cells from G1-to-S phase.

Stimulation of quiescent cells with estradiol also leads to C-myc induction within 30 minutes. Inhibition of c-myc expression by antisense inhibits cell proliferation after estrogen treatment of MCF-7 cells ¹⁰⁵. Inducible overexpression of C-myc leads to activation of G1 Cdks and drives quiescent breast cancer cells into the cell cycle ¹⁰⁶. Thus, C-myc plays an important role in the estradiol-mediated activation of cyclin E-Cdk2 kinase activity.

Antiestrogens cause breast cancer cells to undergo proliferative arrest

Given the potent mitogenic effects of estrogen, a large number of therapeutic antagonists of estrogen signaling have been developed, including antiestrogens. Antiestrogens, such as tamoxifen, and ICI 182780 (Fulvestrant, Faslodex) inhibit estrogen signaling and cause G1 arrest by directly antagonizing the ability of estrogen to bind the ER. Some antiestrogens such as ICI 182780, further inhibit estrogen signaling by reducing cellular ER levels¹⁰⁷. Tamoxifen has been the antiestrogen of choice in the clinical setting for over 25 years. In patients treated for primary breast cancer, the drug provides up to 47 % reduction in the risk of contralateral breast cancer ¹⁰⁸. In patients at high risk of developing breast cancer, tamoxifen, used as a preventative agent, reduced the risk of invasive cancer by up to 49 % ²⁴. Tamoxifen and other antiestrogens cause breast cancer cells to undergo G1 arrest and this appears critical for their therapeutic efficacy. Although antiestrogens may induce both apoptosis and cell cycle arrest, it is the ability of antiestrogens to promote cell cycle arrest rather than apoptosis that predicts response to endocrine therapy ^{109,110}. The antiestrogen Tamoxifen has clinical benefit in some ER positive breast cancers, but not in ER negative tumors ^{108,111}. Although the absence of ER predicts for antiestrogen resistance, loss of ER is not a major mechanism for the development of antiestrogen resistance in ER positive breast cancers; Approximately 30 % of ER positive breast cancers will show initial non-responsiveness to antiestrogens. Unfortunately, ER positive metastatic cancers that do respond to tamoxifen almost invariably become resistant. Tumors that are initially antiestrogen sensitive but progress to a

resistant state do not show loss of ER expression in the majority of cases. *Cell cycle deregulation may be central to the acquisition of an antiestrogen resistant phenotype. This is reviewed in Section II and III of this Final Report.* Such cell cycle defects may not only lead to tamoxifen resistance in breast cancer cell lines but may also contribute to the development of resistance to multiple anti-proliferative stimuli. For example, Herman et al showed that certain tamoxifen resistant breast cancers were resistant to the growth inhibitory effects of both TGF- β and retinoic acid¹¹².

Antiestrogen treatment of breast cancer leads to a decrease in cyclin D1 protein levels and inhibition of Cdk4 and Cdk6 activities. Cyclin D1 downregulation may be essential to the arrest mechanism since in some models, the inducible overexpression of cyclin D1 abrogates the ability of breast cancer cells to maintain cell cycle arrest in the presence of antiestrogens^{113,114}. C-myc is an additional G1 regulator whose deregulation may contribute to antiestrogen resistance. Overexpression of c-myc leads to a failure of MCF-7 cells to maintain growth arrest in the presence of antiestrogens¹¹⁵. Several groups showed that antiestrogen-treatment of ER positive MCF-7 breast cancer cells leads to the accumulation of p21 and p27 in cyclin E-cdk2 complexes and kinase inhibition. *In studies to be presented in Section II, I investigated whether p21 and p27 are required for the anti-proliferative effects of antiestrogens.*

Cross-talk between the ER and MAPK signaling pathways

Although it has long been appreciated that ER acts as a nuclear transcription factor, the activated ER can also activate various signal transduction pathways within minutes of ligand binding in a manner independent of its transcriptional activator function¹¹⁶. Migliaccio et al showed that estradiol activates MAPK activity via an ER-Src/Shc/Ras pathway (see Fig 6 and¹¹⁷). MAPK is a potent mediator of estrogen signaling and drives the progression of cells out of G1 into S phase. Several groups, including our own, have shown that estradiol stimulated cell cycle entry is inhibited by the MEK inhibitors U0126 and PD098059^{118,119}. Although these pathways are normal mediators of

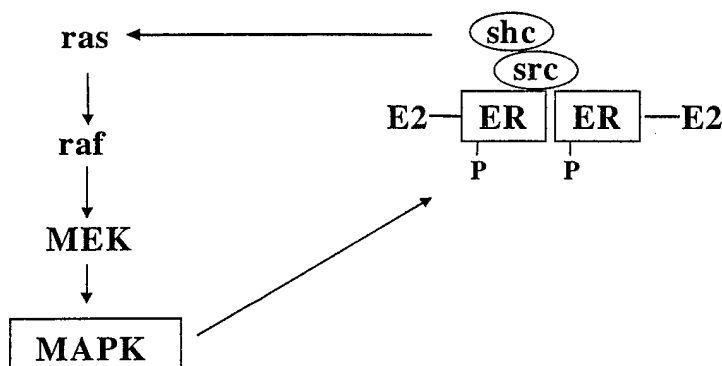


Figure 6. Non-genomic signaling by the ER. In addition to binding DNA and acting as a ligand activated transcription factors, the ER can activate MAPK. MAPK activation involves the binding of Src to the ligand activated ER and activation of Shc-Grb2-Sos-Ras- MAPK signaling.

estrogen signaling, their constitutive hyperactivation in breast cancers may oppose antiproliferative signals that normally cause cell cycle arrest in G1. Hyperactivation of MAPK is observed in 70% of breast cancers, and has been correlated with poor response to tamoxifen therapy and decreased survival ^{120,121}. *In Section III of this report, I tested whether constitutive MAPK activation is a direct cause of antiestrogen resistance in a tissue culture breast cancer model.*

In addition to estradiol, MAPK activity is increased following stimulation of quiescent breast cells with several other mitogens, including EGF, PDGF, IGF-1. These pathways 'cross-talk' with the ER leading to its phosphorylation and ligand independent activation. EGFR activation can cause

MAPK to phosphorylate the ER at Ser 118 in the AF-1 domain *in vitro*^{122,123}. The pp90^{Rsk1} kinase, a downstream target of MAPK, can also phosphorylate and activate ER by phosphorylation at Ser 118 and Ser 167 (^{123,124} and Fig 6).

MAPK activity is essential for G1-to-S phase progression. In MCF-7 breast cancer cells, MAPK signaling may play a role in the estradiol mediated upregulation of cyclin D1 expression^{36,125,126}. Since MAPK has been shown to activate cyclin D1 in fibroblast models, MAPK activation following ligand-mediated ER may also facilitate the transcriptional upregulation of cyclin D1¹²⁷. In fibroblasts, MAPK activation may play an important role in regulating the assembly of active cyclin D1-Cdk4-KIP complexes^{63,128}. In several cancer cell lines and fibroblast models, MAPK signaling regulates the cellular abundance of p27. Ras activation leads to p27 degradation in a MAPK dependent manner in some tissue culture models, and in a PI3K/PKB dependent manner in other cell lines^{68,129}. In addition to affecting p27 levels, active MAPK has been shown to mediate the sequestration of p27 into active cyclin D1-Cdk4 complexes and thereby relieve cyclin E1-Cdk2 inhibition in fibroblasts. *In vitro* phosphorylation of p27 by MAPK was shown to reduce its ability to bind and inhibit cyclin E-Cdk2¹³⁰. However, it remains to be shown whether MAPK is a direct p27 kinase *in vivo*.

MAPK activation in breast cancer may also lead to the phosphorylation and stabilization of c-myc¹³¹, thus facilitating the ability of myc to transcriptionally upregulate several G1 regulators, including cyclin D1 and cyclin D2^{132,133}. Although activating ras mutations are infrequent, Ras overexpression occurs in about 70 % of breast cancers and contributes to altered responsiveness of breast cancer cells to external growth stimuli (for review see¹³⁴). Dickson et al and Sukumar et al showed that oncogenic ras overexpression in MCF-7 cells led to estrogen independence *in vitro*^{135,136}. Ras can activate a number of pathways including MEK-MAPK, and PI3K-PKB (See Fig 7). Ras activates raf-1, a serine threonine kinase that can directly phosphorylate many targets, including

the downstream MAPK kinase (MEK) which in turn can phosphorylate MAPK on threonine and tyrosine. Activated MAPK is a Ser/Thr kinase that can hypophosphorylate several nuclear transcription factors, including myc, elk and pp90^{Rsk}.

Deregulated receptor tyrosine kinase signaling is associated with MAPK activation and antiestrogen resistance

Although estrogens drive cell proliferation via the activation multiple pathways including the MAPK pathway many ER positive breast cancers no longer depend on estrogens to proliferate. The establishment of estrogen independent proliferation by breast cancer cells may occur via the upregulation of signaling pathways that mimic estrogen signaling. Not surprisingly, many such pathways activate MAPK signaling, and constitutive activation of many of these pathways may contribute to antiestrogen resistance.

MAPK activation in human breast cancer may result from oncogenic activation of upstream receptor tyrosine kinase signaling and may alter the response of cells to estrogens and antiestrogens. *In the following discussion, I briefly review how deregulated signaling via the epidermal growth factor receptor (EGFR) and HER-2/ErbB2 may contribute to constitutive MAPK activation and antiestrogen resistance in breast cancer.* There are four ErbB receptor tyrosine kinases that may be expressed by normal and malignant breast epithelial cells, ErbB1 (EGFR), ErbB2 (HER-2/neu), ErbB3 and ErbB4. Activation of any of the ErbB receptor family members may lead to homo- or heterodimerization with other ErbB family members although ErbB2 has been shown to be the preferred heterodimerization partner for all activated ErbB family members¹³⁷. Once activated, ErbB receptors become phosphorylated on tyrosines, and these phosphotyrosines serve as docking sites for SH2 domain containing effector proteins¹³⁸, which in turn may stimulate Ras activation.

About 30 % of breast cancers overexpress EGFR and this is associated with poor prognosis, loss of the ER¹³⁹ and loss of estrogen responsiveness^{140,141}. Several studies have shown that EGFR

overexpressing MCF-7 lines are antiestrogen resistant^{142,143} and patients with cancers expressing high levels of EGFR are less likely to respond to hormonal therapy. The long term culture of MCF-7 cells with the antiestrogen ICI 182780 led to increased EGFR and ErbB2 expression and increased TGF- α secretion¹⁴⁴. Ligand independent ER activation via constitutive EGFR activity could theoretically contribute to antiestrogen independence. However, *in vivo*, the upregulation of ErbB receptors does not appear to occur as an adaptive response in patients treated with tamoxifen. Newby et al showed that there was no change in expression of ErbB2 or EGFR in patients before and after treatment with tamoxifen¹⁴⁵, and that low expression levels of ErbB2 and EGFR prior to initiating treatment was sufficient to predict tamoxifen effectiveness. Thus, acquired overexpression of ErbB receptors during Tamoxifen treatment is unlikely to be major mechanism of antiestrogen resistance.

The ErbB2/neu/HER-2 receptor is overexpressed in 10-35 % of breast carcinomas and associated with poor prognosis^{146,147}. Although HER-2 overexpression can mediate antiestrogen resistance in tissue culture and *in vivo* mouse models, there are conflicting reports regarding the relationship between HER-2 status and hormone sensitivity. Some groups have shown no evidence for poorer response of HER-2 patients to tamoxifen in patients with advanced disease^{148,149}. However, others showed a strong correlation between HER-2 overexpression and ER loss¹⁵⁰. Upregulation of ErbB2 signaling in MCF-7 by transfection of ErbB2 leads to tamoxifen resistance¹⁵¹⁻¹⁵³. ERB2 overexpression activates MAPK¹⁵⁴⁻¹⁵⁶). Two recent studies have shown a correlation between HER-2 overexpression and reduced p27 levels in primary human breast cancers^{157,158}. *In Section III, I show that constitutive MAPK activation resulting from MEK or HER-2 overexpression mediate antiestrogen resistance, at least in part, through functional inactivation of p27.*

CELL CYCLE ARREST BY TGF- β

In Section V, I describe studies we undertook following our observation that p27 was essential for G1 arrest by antiestrogens. Specifically, we investigated the role of p27 in the cell cycle arrest

by Transforming growth factor- β (TGF- β). Like antiestrogen resistance, resistance to TGF- β is common in malignant breast cancer cells. Normal breast cells however are exquisitely sensitive to TGF- β . Although not in the original statement of work, we undertook these studies alongside our studies of p27 in antiestrogen resistance. We are pleased to report that these studies show that p27 is a key effector of G1 arrest not only in antiestrogen treated breast cancer cells, but also in malignant breast cancer cell as well. TGF- β mediates G1 cell cycle arrest by inducing or activating Cdk inhibitors, and by inhibiting factors required for Cdk activation. The following reviews general mechanisms contributing to cell cycle arrest by TGF- β .

TGF- β is a potent inhibitor of mammary epithelial cell proliferation^{159,160} and regulates mammary development *in vivo*^{161,162}. Mammary specific overexpression of TGF- β in transgenic mice can induce mammary hypoplasia and inhibit tumorigenesis^{163,164}. While normal human mammary epithelial cells (HMEC) are exquisitely sensitive to TGF- β ¹⁶⁵, human breast cancer lines require 10-100 fold more TGF- β to produce an antimitogenic response and some show complete loss of this effect¹⁶⁶. While loss of growth inhibition by TGF- β in human cancers can arise through loss of TGF- β production or through mutational inactivation of the TGF- β receptors and Smad signaling molecules see¹⁶⁷, these defects are not observed in most arrest-resistant cancer lines. This observation, and the frequent appearance of resistance to more than one inhibitory cytokine in human tumors¹⁶⁸, underline the importance of the cell cycle effectors of growth arrest by TGF- β as targets for inactivation in cancer.

TGF- β inhibits phosphorylation of the retinoblastoma protein

Cells are sensitive to TGF- β during a discrete period in early G1, until they reach the "restriction point" 6-10 hours following G0 release^{169,170}. When TGF- β is added after this critical time point, cells complete the cell cycle, but arrest in the subsequent G1 phase. Laiho et al observed

that TGF- β inhibits pRb phosphorylation when it is added in early G1 ¹⁶⁹. This key observation suggested that TGF- β was acting prior to the G1/S transition to inhibit an Rb kinase and led to the investigation of TGF- β effects on cell cycle regulators ^{30,170-172}. These studies have shown that TGF- β prevents or inhibits G1 cyclin-Cdk activation through multiple mechanisms leading to pRb dephosphorylation (see Figure 1). E2F activity is also impaired by TGF- β through a decline in E2F mRNA levels ¹⁷³. The observation that E2F overexpression can prevent TGF- β mediated arrest, underlines the importance of TGF- β 's effects on pRb and E2F ¹⁷³.

TGF- β downregulates c-myc

In many cell types, TGF- β causes a rapid inhibition of *c-myc* transcription ^{160,174,175}. Transcriptional regulation by the c-myc protein is required for G1-to-S phase progression. Downregulation of *c-myc* by TGF- β is believed to be important for arrest, since *c-myc* overexpression causes TGF- β resistance ^{160,176}. Repression of the *c-myc* gene by TGF- β may directly or indirectly contribute to the loss of G1 cyclins ^{177,178}, to downregulation of Cdc25A ¹⁷⁹ and to the induction of the Cdk inhibitor, p15 ¹⁸⁰.

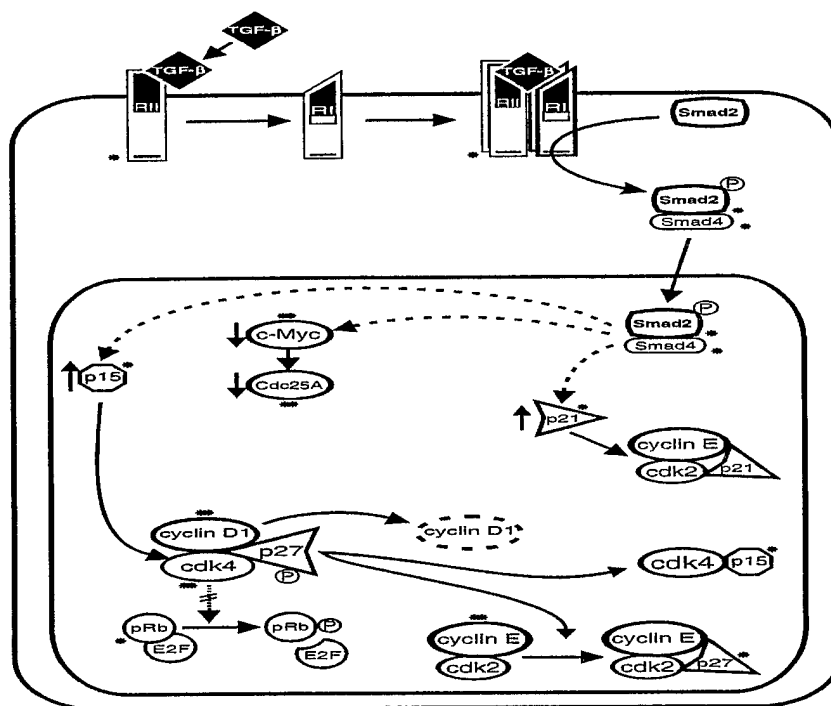


Figure 7. Mechanisms of cell cycle arrest by transforming growth factor (TGF)- β and their deregulation in cancer. TGF- β receptor activation leads to Smad2 phosphorylation. Phosphorylated Smad2 then binds Smad4 and the Smad2-Smad4 complex translocates to the nucleus to modulate transcription. Although p15 and p21 genes are induced and c-myc and Cdc25A repressed by TGF- β , these may not be direct effects of Smad2-Smad4 action (dotted lines). TGF- β inhibits G₁ cyclin-cyclin-dependent kinases (Cdks) by increasing p27 (+/-p21) binding to cyclin E-Cdk2, thereby inhibiting retinoblastoma protein (pRb) phosphorylation. *Components of the TGF- β effector pathway that are mutated and/or functionally inactivated in human cancers; **molecules whose activation or overexpression may contribute to TGF- β arrest resistance.

Effects on G1/S cyclins

TGF- β causes loss of G1 cyclins in a cell type dependent manner. Cyclin A expression is downregulated by TGF- β in most cell types^{171,181} and a TGF- β regulated region of the cyclin A promoter has been identified¹⁸². Effects of TGF- β on cyclin E differ among different cell lines. For example, in HaCat keratinocytes, TGF- β decreases both mRNA and protein levels of cyclin A and cyclin E, while in HMEC, cyclin E mRNA is reduced but protein levels are not^{171,181}. Although cyclin D1 levels are decreased by TGF- β in some cell types, this usually occurs late as a consequence of arrest^{183,184}.

Cooperation between p15 and p27

In epithelial cells, including HMECs, the INK4 and the KIP proteins collaborate to inhibit D-type cyclin- and E-type cyclin-Cdks to bring about G1 arrest by TGF- β ^{51,51,185}. *p15^{INK4B}* was first cloned as a gene upregulated by TGF- β ⁵⁰ and its induction involves an Sp1 site in the promoter¹⁸⁶. TGF- β induces *p15^{INK4B}* and stabilizes the p15 protein, leading to p15 binding and inhibition of Cdk4 and Cdk6. Cyclin D1 and KIP molecules dissociate from Cdks 4 and 6, and p27 accumulates in cyclin E-Cdk2 complexes inhibiting the latter^{51,185}. A late downregulation of cyclin D1 and Cdk4 follows G1 arrest. TGF- β appears to actively regulate p27's affinity for its targets, independent of p15 function, favoring p27 accumulation in cyclin E complexes⁵¹.

Effects on Cdk2 phosphorylation

TGF- β also regulates Cdk2 phosphorylation. In Mv1Lu cells, TGF- β inhibits Cdk2 in part by inhibiting phosphorylation on Thr 160^{171,172}. p27 can inhibit CAK access to cyclin bound Cdks *in vitro*¹⁹². Thus, TGF- β may prevent CAK action by increasing the binding of p27 to cyclin E-Cdk2. However, in HepG2 cells, TGF- β inhibits the enzymatic activity of Cak1p⁵, indicating an alternative mechanism for TGF- β 's inhibition of Thr 160 phosphorylation of Cdk2.

Dephosphorylation of inhibitory Cdk sites by Cdc25A is required for G1 progression cyclin E-bound Cdk2 and is required for G1-to-S phase progression¹⁹³. In a human breast epithelial line, TGF- β reduced *Cdc25A* expression associated with an increase in inhibitory Cdk phosphorylation¹⁷⁹. The effect on *Cdc25A* expression may be secondary to TGF- β 's repression of *c-myc*, since in some cell types, *Cdc25A* is induced by *c-myc*⁸.

LOSS OF TGF- β MEDIATED G₁ ARREST IN CANCER

In non-transformed epithelial cells, TGF- β causes G1 arrest through downregulation of *c-myc*, inhibition of the G1 Cdks and hypophosphorylation of pRb. Overlapping or redundant cell cycle controls assure growth arrest. However, in malignantly transformed cells, this redundancy is

often lost and carcinoma-derived cells are usually refractory to growth inhibition by TGF- β ¹⁶⁶. Indeed, in advanced cancers, TGF- β may promote tumor growth and metastatic progression. In this section, we review how deregulation of many different cell cycle mechanisms abrogate TGF- β arrest in cancer (see Figure 2).

Altered Cdk inhibitor expression and function

Deregulation of the INK4 family may contribute to TGF- β resistance in cancer. In human tumors, deletion of *p15* often accompanies *p16* deletion due to their proximity on chromosome 9p.¹⁹⁴⁻¹⁹⁶ Silencing of *p15* through promoter hypermethylation, observed in leukemias, is associated with loss of TGF- β sensitivity ^{197,198}. However, in other TGF- β resistant cells, p15 protein levels may increase normally, indicating that, at least in these lines, a functional p15 is not sufficient to mediate arrest by TGF- β ¹⁸⁸.

While p15 and p27 cooperate to inhibit the G1 cyclin-Cdks in normal cells, neither of these Cdk inhibitors is essential for G1 arrest by TGF- β . p15 is clearly not essential for TGF- β -mediated G1 arrest, since cells bearing *p15* deletions can respond through upregulation of p21 and p27 ^{184,188}; or downregulation of *cdc25A* ¹⁷⁹. Lymphocytes from p27 null mice can still arrest in response to TGF- β ³⁹. Although TGF- β leads to an increase in cyclin E-cdk2 bound p27 in a vast majority of cell types, it is not clear whether p27 is required for TGF- β -mediated cell cycle arrest. *In studies to be presented in Section V of this Final Report, I show that the requirement for p27 in G1 arrest by TGF- β differed between normal and transformed (malignant) breast cells. In normal cells, multiple redundant pathways cooperate to mediate arrest, but in cancer cells, the progressive loss of other checkpoints may make p27 indispensable for TGF- β mediated G1 arrest.* Thus, altered p27 regulation may contribute to TGF- β resistance in cancer cells.

c-myc activation and TGF- β resistance

TGF- β arrest resistant cells often fail to downregulate *c-myc*¹⁸⁸. Moreover, oncogenic activation of *c-myc*, seen in a number of human malignancies including breast cancer, may impair TGF- β responsiveness through several or all of the mechanisms discussed in earlier sections of this section.

c-myc overexpression may increase G1 cyclin levels. *c-myc* may indirectly regulate the expression of cyclins D1, E and A^{177,178}. *c-myc* induction of cyclin D1 or D2 may lead to the sequestration of p27 and p21 away from cyclin E/Cdk2 and thus contribute to cyclin E-Cdk2 activation^{132,133}. However, these effects, best demonstrated in fibroblast lines, may not be relevant to TGF- β resistance. In Mv1Lu cells, *c-myc* overexpression prevents arrest by TGF- β in part by inhibiting p15 induction¹⁸⁰. *c-myc* effects on D-type cyclin expression and cyclin D/Cdk4 complex formation were not sufficient to account for loss of the TGF- β response. Thus, repression of *p15* by *c-myc* may be important in the arrest resistant phenotype.

Additional mechanisms link *c-myc* with cyclin E-Cdk2 activation. Overexpression of *c-myc* can induce a heat labile factor that binds p27 and inhibits its association with cyclin E-Cdk2⁵⁵. This effect is independent of p27 degradation. Although in some cell types, cyclin D1 and D2 may be the *c-myc*-induced inhibitors of p27^{132,206}, in other models, the *c-myc* induced inhibitor of p27 appears to be a protein other than D-type cyclins⁵⁵.

Oncogenic activation of *c-myc* may lead to *Cdc25A* overexpression and loss of TGF- β mediated repression of *Cdc25A*¹⁷⁹. Overexpression of *Cdc25A* is observed in primary breast cancers and is associated with a poor patient prognosis. The increased *Cdc25A* may represent one of the checkpoints whose disruption makes subsequent disruption of p27 function more critical during breast cancer progression.

Activation of ras and its effector pathways and TGF- β resistance

Overexpression of activated *ras* has been shown to abrogate the antimitogenic effects of TGF- β ²⁰⁷. Mutational activation of *ras* is common in many human cancers and may be linked to TGF- β resistance through a number of mechanisms. Activated Ras can interfere with TGF- β signaling by altering Smad2 phosphorylation and signal transduction ²⁰⁸. Moreover, Ras activation can increase cyclin D1 levels through both transcriptional and post-translational mechanisms ^{63,209,210}. Ras activation also accelerates p27 degradation ^{68,130}, and in some models also requires co-expression of Myc ⁶⁷. Oncogenic activation of different *ras* effector pathways may abrogate p27 function ^{68,211}, contributing importantly to TGF- β resistance.

In summary, TGF- β induced G1 arrest occurs through induction of *p15* and *p21* genes, repression of the *c-myc*, *Cdc25A*, *cyclin E* and *A* genes and an increase in the association of p15, p21 and p27 with target Cdks. Inactivation of G1 cyclin-Cdks leads to pRb dephosphorylation and E2F inhibition. These mechanisms are summarized in Figure 7.

Section II (BODY)

**Downregulation of p21^{WAF1/Cip1} or p27^{Kip1} abrogates antiestrogen mediated
cell cycle arrest in human breast cancer cells**

A version of this section is published in Proceedings of
the National Academy of Sciences with the DOD awardee as co-first author and is
appended in appendix III

SUMMARY OF DATA REPORTED IN LAST YEAR'S ANNUAL REPORT TO DOD

Last year I reported that in MCF-7 breast cancer cells, estrogen stimulated cell cycle progression through loss of kinase inhibitor proteins (KIPs), p27 and p21, and G1 cyclin-Cdk activation. Treatment with antiestrogen drugs, Tamoxifen or ICI 182780, caused cell cycle arrest, with up-regulation of both p21 and p27 levels, an increase in their binding to cyclin E-Cdk2 and kinase inhibition. The requirement for these KIPs in the arrests induced by estradiol depletion or by antiestrogens was investigated using antisense. Antisense inhibition of p21 or p27 expression in estradiol-depleted or antiestrogen-arrested MCF-7 led to abrogation of cell cycle arrest, with loss of cyclin E-associated KIPs, activation of cyclin E-Cdk2 and S phase entrance. These data demonstrate that depletion of either p21 or p27 can mimic estrogen-stimulated cell cycle activation and indicate that both of these KIPs are critical mediators of the therapeutic effects of antiestrogens in breast cancer. These studies were reported in last year's Annual Report.

SPECIFIC INTRODUCTION TO SECTION II

Estradiol is mitogenic in up to 50% of *de novo* breast cancers, causing recruitment of quiescent cells into G1 and shortening the G1-to-S phase interval ^{212,213}. While 70% of breast cancers express the estrogen receptor (ER), only two thirds of these will respond to antiestrogens, of which, Tamoxifen is the most widely used ^{214,215}. Antiestrogens, such as Tamoxifen, its active metabolite, 4-hydroxytamoxifen (4-OH TAM) and the more potent steroidal antiestrogen ICI 182780 (Faslodex) lead to a G0/G1 arrest in susceptible ER positive breast cancer cells ²¹⁶⁻²¹⁹. Unfortunately, hormonally responsive breast cancers invariably develop resistance to antiestrogens despite the continued expression of wild type ER in most cases ²²⁰⁻²²³. Estrogens induce conformational changes in the ER, which promote its nuclear localization, dimerization and function as a ligand activated transcription factor ²²⁴⁻²²⁶. In addition, ligand binding to the ER can rapidly and transiently activate signal transduction pathways, notably the mitogen-activated protein kinase (MAPK) in breast cancer and in other cell types ^{117,227}. Since antiestrogen resistance usually develops in the presence of an intact ER, the mechanisms whereby ER modulates the cell cycle may be altered during breast cancer progression. The evolution of prostate cancer to hormone independence also occurs without loss of the androgen receptor ^{228,229} and may reflect a common mechanism of cell cycle misregulation.

Progression through the cell cycle is governed by a family of cyclin dependent kinases (Cdks), whose activity is regulated by phosphorylation ⁴, activated by cyclin binding ^{2,11} and inhibited by the Cdk inhibitors of the INK4 family (p16^{INK4A}, p15^{INK4B}, p18, p19) and KIP family (p21^{WAF-1/CIP-1}, p27^{Kip1}, and p57^{KIP2}) ^{11,230}. Passage through G1 into S phase is regulated by the activities of cyclin D-, cyclin E- and cyclin A-associated kinases. Although p27 protein is strongly expressed in normal mammary epithelial tissue, decreased levels of p27 protein in

primary breast cancers are correlated with poor prognosis^{231,232} and steroid independence²³¹. Reduced p21 levels have also been associated with a poor prognosis in some breast cancer studies^{87,233,234}. Expression of the ER, a good prognostic factor in breast cancer, is associated with higher levels of both p21 and p27 proteins^{231,233-235}. Our observation that loss of p27 was strongly associated with hormone independence²³¹ stimulated our investigation of the role of these KIPs in cell cycle effects of estrogen and antiestrogens in breast cancer cells.

While recent reports correlate estrogenic stimulation with activation of cyclin E-Cdk2, some suggest the importance of the Cdk inhibitor p21^{236,237} and others emphasize a role for p27²³⁸. An understanding of how estrogens and antiestrogens influence the cell cycle and the mechanisms of their alteration in cancer progression may facilitate the development of new hormonal treatments for breast cancer and other hormone dependent cancers. The present study provides evidence that both p21 and p27 play essential roles in the cell cycle arrest of breast cancer cells by antiestrogens.

How does estradiol affect the cell cycle profile of MCF-7

We found that estradiol stimulates a shift of KIP proteins from cyclin E-Cdk2 into cyclin D1-Cdk4. Estradiol stimulation of steroid deprived, quiescent MCF-7 breast cancer cells induced synchronous cell cycle re-entry. S phase entrance was detected by 12 hours, with the peak % S phase cells at 24 hours (Fig. 1A).

How do the levels of cell cycle regulator change following addition of estradiol?

The levels of cyclin E, Cdk2, and Cdk4 remained constant and p15^{INK4B} protein levels fell as cells moved into G1. MCF-7 cells do not express p16^{INK4A} due to deletion of this gene²⁴³. Cyclin D1 was not detected in quiescent cells, but rose within 3 hours of estradiol addition and

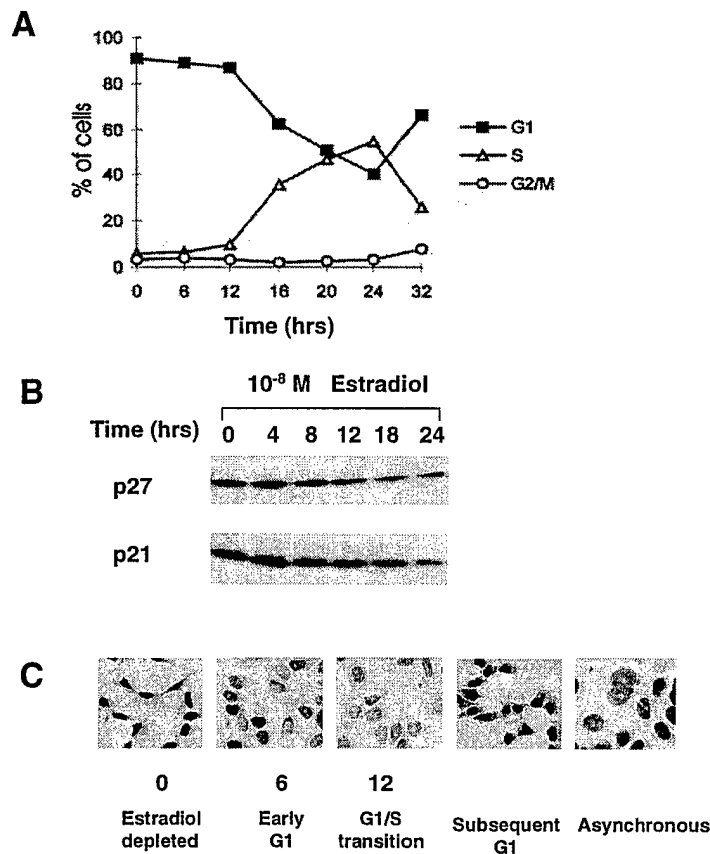


Figure 1. Losses of p21 and p27 during estradiol stimulation of quiescent MCF-7 cells. Quiescent, estradiol depleted MCF-7 cells were stimulated by re-addition of 10 nM estradiol and samples taken at intervals thereafter. (A) Cell cycle synchrony was determined by dual BrdU /PI pulse labeling and flow cytometric analysis. (B) p21 and p27 immunoblots revealed levels of these proteins during cell cycle progression. (C) p27 protein was assayed by immunohistochemistry in asynchronous cultures and at the indicated times (hrs) after estradiol stimulation of quiescent MCF-7 as described ²³¹.

remained constant thereafter. p21 and p27 protein levels fell by three and five fold, respectively by 24 hours (Fig. 1B). Immunohistochemical analysis of p27 supported the immunoblotting data (Fig. 1C). Estradiol depleted, quiescent MCF-7 cells showed strong nuclear p27 staining that was notably reduced by 6 hours after addition of estradiol and barely detectable above background by 12 hours as S phase entrance began.

How does the binding of p21 and p27 to cyclin E-cdk2 and cyclin D-cdk4 change during estradiol mediated G1-S progression?

The pattern of binding of p21 and p27 to Cdk4 and Cdk2 complexes differed during estradiol stimulated cell cycle progression in MCF-7. Cdk4 bound p27 was abundant in estradiol-depleted cells and increased in parallel with the increased assembly and activation of cyclin D1-Cdk4 between 3 to 9 hours after estradiol addition (Fig. 2A and B). Cyclin D1-dependent kinase activities and cyclin D1 binding to Cdk4 were reduced by 12 hours and undetectable by 16 hours. Although p21 protein was elevated, very little p21 was detectable in Cdk4 complexes in quiescent MCF-7. As for p27, cyclin D1-Cdk4 assembly was accompanied by increased p21 binding, in keeping with the function of p21 and p27 as positive regulators of cyclin D1-Cdk4 assembly^{46,47}. While cyclin D2 and Cdk6 are detectable in MCF-7, cyclin D1-Cdk4 has been shown to be the major D-type cyclin dependent kinase in these cells^{236,237}. In contrast to their pattern in cyclin D1 complexes, activation of cyclin E-Cdk2 following estradiol stimulation was correlated loss of p27 and p21 from cyclin E-Cdk2. Cyclin E-Cdk2 activation was correlated with S phase entrance (Fig 2B and C).

How do the cell cycle regulators change following antiestrogen treatment?

p21 and p27 bind and inhibit cyclin E-Cdk2 on interruption of mitogenic ER signaling. To further investigate cell cycle regulation by estrogen, ER signaling was interrupted in asynchronous MCF-7 cultures in three ways: by treatment with the pure ER-antagonist ICI 182780, by the addition of antiestrogen 4-OH TAM, or by steroid depletion. All induced quiescence, with the S phase fraction falling from 29-36% to 1-5% over 48 hours with a corresponding increase in the % cells in G0/G1 phase (data shown for ICI 182780 in Fig. 3). p21 and p27 proteins increased, as did their binding to cyclin E-Cdk2, in parallel with kinase inhibition (Fig. 3B). Although levels of cyclin E-bound Cdk2 were unchanged, there was an accumulation of the slower-mobility, non-CAK activated Cdk2, lacking threonine 160 (Thr160) phosphorylation. The pattern of increase in p21 and p27 and of their binding to cyclin E during estradiol depletion and 4-OH TAM arrest were similar to that shown for ICI 182780 in Fig 3B. The loss of cyclin D1 observed during antiestrogen treatment would lead to dissociation of KIPs from cyclin D1 complexes and foster KIP inhibition of cyclin E-Cdk2^{244,245}.

Are p21 and p27 essential for G1 arrest by antiestrogens in MCF-7?

These data and earlier work support the notion that estrogens and antiestrogens work through changes in p21 and p27 levels. Foster et al showed that p27 immunoprecipitation depleted significantly cyclin E-Cdk2 inhibitory activity from serum and amino-acid starved MCF-7 cells²³⁸. Others showed that most of the cyclin E-Cdk2 inhibitory activity in Tamoxifen or ICI 182780-arrested MCF-7 was removed by immunodepletion of p21. However, immunodepletion of both p21 and p27 was required to fully deplete cyclin E from arrested cells, indicating that cyclin E is bound to either p21 or p27 in an ER-blocked arrest state^{236,237,244}. These authors proposed that the estradiol-stimulated up-regulation of cyclin D1 served to sequester p21 away from cyclin E complexes leading to activation of cyclin E and pRb

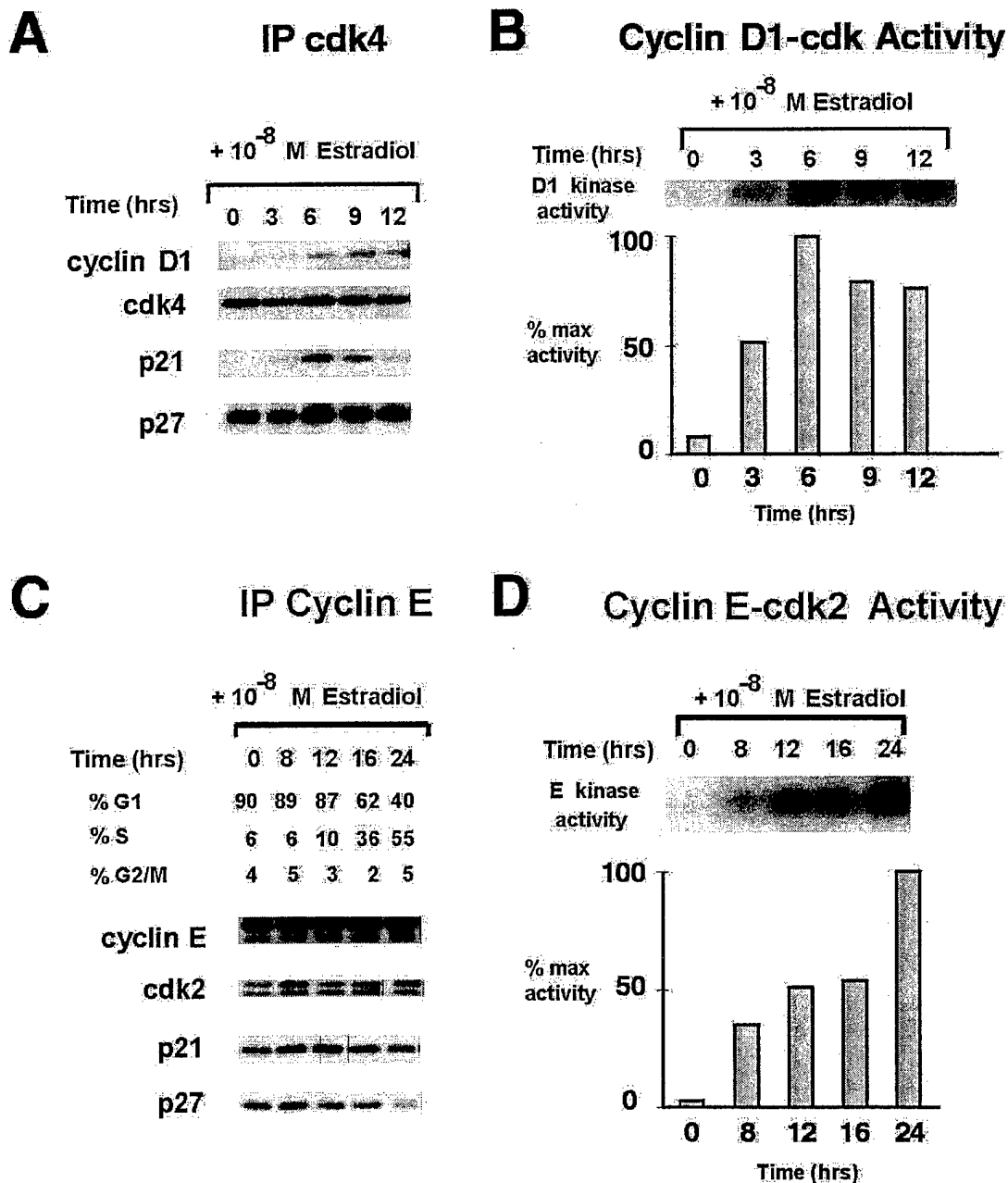


Figure 2. Different patterns of KIP binding during cyclin D1-Cdk4 and cyclin E-Cdk2 activation. Cdk4 (A) and cyclin E (C) immunoprecipitates (IP) from cell lysates recovered at intervals after re-addition of estradiol to steroid-depleted MCF-7 cells were resolved and analyzed by immunoblotting with the indicated antibodies. Cyclin D1 (B) and cyclin E (D) immunoprecipitates were assayed for kinase activity ²⁵⁷ at intervals (hrs) following estradiol stimulation of quiescent MCF-7.

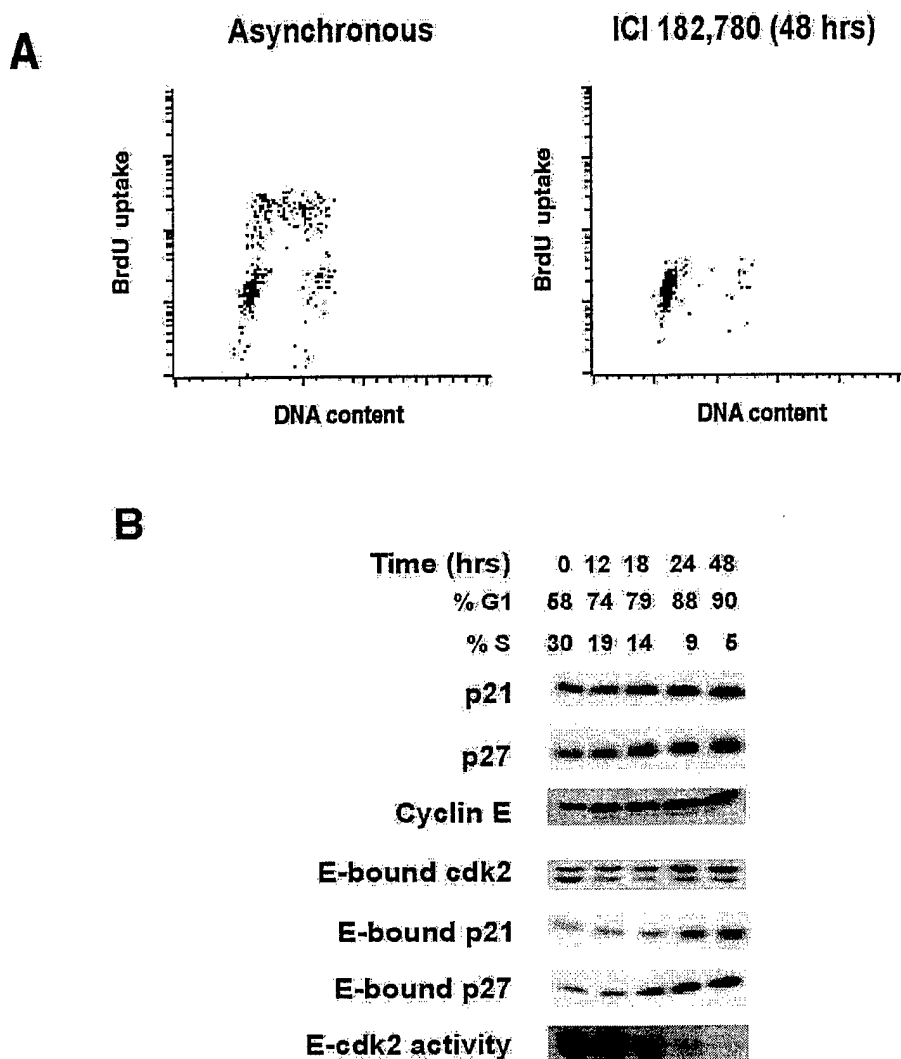


Figure 3. p21 and p27 proteins increase during G0/G1 arrest by ER blockade. Asynchronously growing MCF-7 cells were treated with the ER blocking drug ICI 182780 (Faslodex) at time 0 hrs and samples collected for flow cytometry or protein analysis at times indicated (hrs). (A) Cell cycle distribution before and 48 hrs after drug treatment. (B) Lysates were analysed by immunoblotting with the indicated antibodies. Cyclin E immunoprecipitates were immunoblotted for associated p21 or p27 or analyzed for associated histone H1 kinase activity as in ²⁵⁷. Similar results were obtained for arrests with 4-OH TAM or following transfer to estradiol-depleted, charcoal stripped serum.

phosphorylation. However, none of these earlier studies definitively established a requirement for p21 and p27 in cell cycle arrest by antiestrogens. To test directly whether p21 and p27 play essential roles in the cell cycle arrest following blockade of ER signaling in MCF-7 cells, we used antisense oligonucleotides to inhibit p21 (ASp21) or p27 (ASp27) expression in cells arrested by ICI 182780, 4-OH TAM or estradiol depletion (data shown for ASp27 in estradiol depleted MCF-7 in Fig 4). A G-clamp heterocycle modification, a cytosine analog that clamps on to a guanine, was designed to enhance antisense/RNA interaction and showed increased antisense oligonucleotide potency over the C5 propynyl-modified oligonucleotides used in earlier assays^{241,242}. Within 1 hour of transfection, ASp27 treated cells showed a four-fold reduction in p27, but no loss of p21 (Fig 4A) and p27 levels reached a nadir at about 6 hours post-transfection. The ASp21 oligos showed a similar specificity with no immediate loss of p27. Metabolic pulse labeling of ASp27 transfected cells showed specific inhibition of p27 synthesis but no effect on p21 protein synthesis at 1 and 21 hours after completion of the transfection (Fig 4A).

Treatment with ASp27 lead to hyperphosphorylation of pRb and p130, CAK phosphorylation at Thr160 on Cdk2 (Fig 4B), loss of p27 binding to cyclin E-Cdk2 and Cyclin E-Cdk2 activation (Fig 4C and D), all consistent with stimulation of G1-to-S phase progression. Similar findings were observed for ASp21 treated cells. ASp21 treatment of cells arrested by estradiol-depletion, 4-OH TAM, or ICI 182780 showed loss of p21 and loss of p21 from cyclin E-Cdk2 with activation of this kinase accompanying S phase entry (not shown). These effects were not observed in the MSM and lipid control groups. Results shown are representative of up to 3 repeat experiments. It is notable that ASp27 treated cells showed late downregulation of p21 at 21 hours post-transfection. Metabolic labeling of ASp27 transfected cells showed

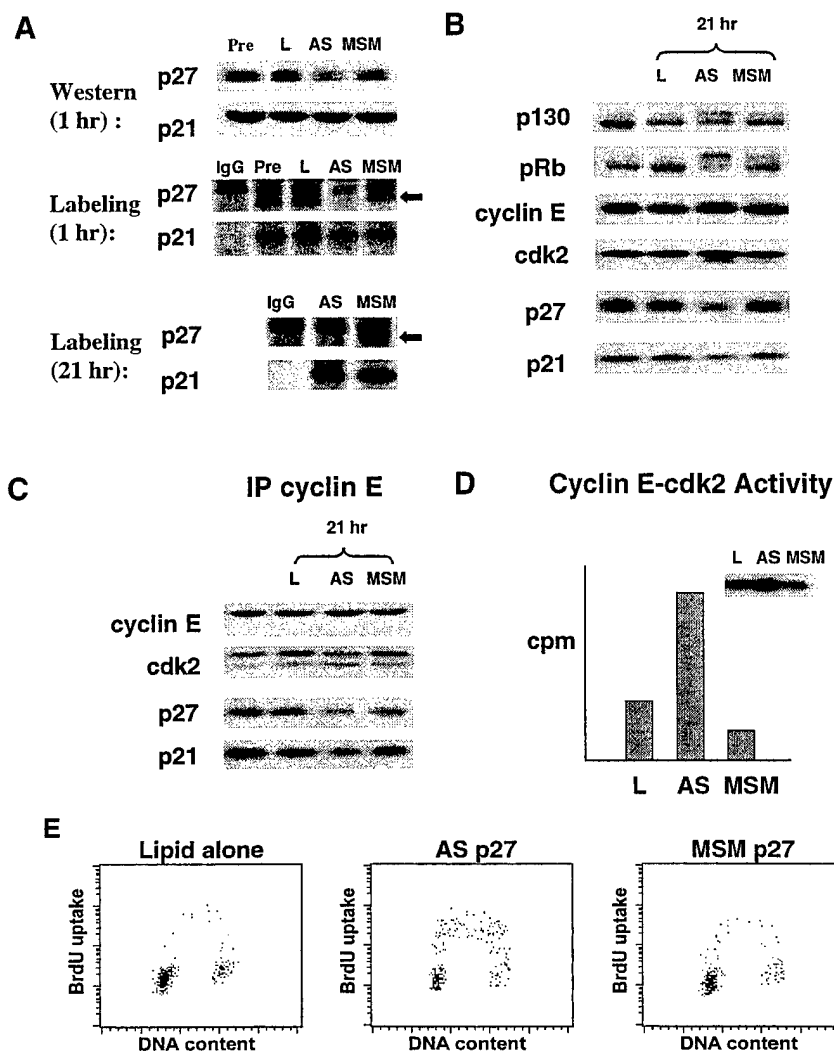


Figure 4. Requirement of p27 for cell cycle arrest by estradiol depletion. (A) Estradiol-depleted MCF-7 cells were lysed before (left lane) or one hour after exposure to lipid only (L), ASp27 (AS) or MSMp27 (MSM) and immunoblotted for p21 and p27. Before and at 1 and 21 hours after ASp27 transfection, cells were metabolically pulse labeled with 35 S-methionine and p21 and p27 immunoprecipitated from lysates containing equal TCA incorporation. The position of metabolically labeled p27 is indicated by an arrow. A non-specific band, migrating close to p27 was present in all lanes, including the control non-specific IgG lane. (B) Immunoblotting shows cell cycle regulatory protein levels before (left lane) or 21 hours after transfection of lipid alone (L), ASp27 or MSMp27. Cyclin E immunoprecipitates (IP) were recovered from the same lysates as in B above and (C) immunoblotted to detect associated proteins or (D) assayed for cyclin E associated histone H1 kinase activity. (E) Flow cytometry 21 hours after transfection with ASp27, lipid alone or MSMp27.

persistent specific inhibition of p27 synthesis at 21 hours, but no inhibition of p21 synthesis as cells were entering S phase. Thus the reduction of p21 protein is not due to inhibition of p21 synthesis by the ASp27 oligo. Rather, the ASp27-induced inhibition of p27 synthesis was sufficient to move cells out of quiescence and the subsequent loss of p21 most likely reflects the changes in post-translational regulation of p21 leading to its degradation at the G-to-S phase transition²⁴⁶. Data in Fig 4 A-C, for ASp27 treatment of ICI 182780-arrested cells, are similar to results for ASp27 treatment of cells arrested by estradiol depletion or 4-OH TAM (not shown). Despite the continued blockade of ER signaling, ASp21 or ASp27 transfection stimulated cell cycle entry of cells arrested by steroid depletion, 4-OH TAM, or ICI 182780, with an S phase fraction of 21%-26 % at 21 hours (or 15 hours after reaching minimum levels of the AS-targeted KIP protein, Figs 4E and 5) and 29-36 % at 28 hours post-transfection. Thus, ASp21 or ASp27 was sufficient to mimic the effect of estradiol on G1-to-S phase progression in MCF-7 cells. These data indicate that a key effect of ER signaling is to relieve KIP-mediated inhibition of Cdk2.

ASp27 not only inhibits p27 synthesis, but would also lead to an increase in p27 degradation. As cyclin E-Cdk2 is liberated from bound p27, it then phosphorylates remaining p27 molecules on Thr187, accelerating thereby p27 degradation^{40,41,247}. Moreover, the activation of cyclin E-Cdk2 is autocatalytic through activation of Cdc25A by cyclin E-Cdk2^{6,7}. Thus, a relatively small initial reduction in p27 can stimulate a significant loss of p27 from cyclin E complexes.

While breast cancer cells arrested by interruption of ER signaling have high levels of both p21 and p27, only p27 is elevated in serum starved fibroblasts and p27 is essential for arrest by serum starvation in immortalized fibroblasts^{35,248}. Although p21 can compensate in part for the lack of p27 in serum starved p27-null MEFs²⁴⁹, the mechanisms of quiescence induced by

serum starvation in fibroblasts and by steroid depletion in malignant breast epithelial cells differ importantly. Our data demonstrate that the cell cycle arrest induced by estradiol depletion or ER blockade requires both p21 and p27 and that these KIPs are not merely upregulated as a consequence of cell cycle arrest.

Mitogenic effects of estradiol include the upregulation of cyclin D1 though increased transcription^{244,245} and stabilization of cyclin D1 protein by assembly with Cdk4 and Cdk6^{250,251}, the latter mediated by p21 and p27^{46,47}. In addition, the present data establish definitively that estradiol-mediated losses of p21 and p27 relieve the inhibition of cyclin E-Cdk2^{236,237,244}.

In estradiol-depleted MCF-7, although p21 and p27 were abundant, they were not competent to stabilize cyclin D1 via assembly with its Cdk partners. Cyclin D1 synthesis is

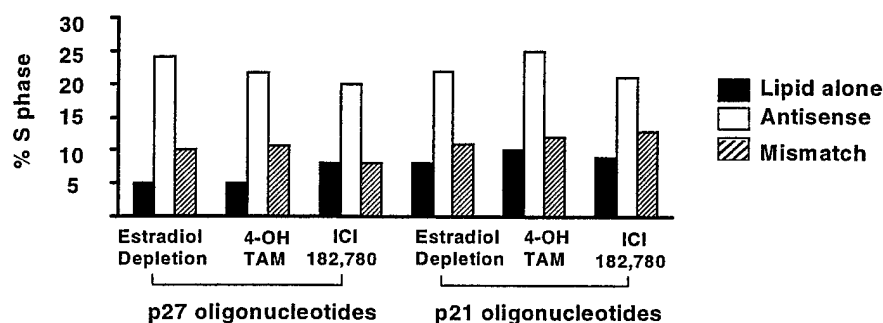


Figure 5. Requirement for p21 and p27 in G1 arrest by ER blocking drugs or estradiol-depletion. MCF-7 cells were arrested by estradiol depletion or by treatment with 4-OH TAM or ICI 182780. The graph indicates the percent S phase cells after 21 hours exposure to lipid only (black bars), antisense (white bars) or mismatch (hatched bars) oligonucleotides to either p21 or p27.

detectable in metabolically labeled quiescent breast epithelial cells, but its association with Cdk4 or Cdk6 is only detectable several hours after mitogenic stimulation (⁵¹ and S. Cariou and J. Slingerland, unpublished results). Thus, an important effect of estradiol may be the conversion of p21 and p27 from a form that does not support assembly of cyclin D-Cdk complexes in G0, to one that does. Similarly, in serum-starved fibroblasts, p27 did not support cyclin D1-Cdk4 complex formation even after ectopic cyclin D1 overexpression ²⁵². Moreover, in p21/p27 null cells, overexpression of cyclin D1 did not permit its assembly with Cdk4 ⁴⁷. Following estradiol stimulation in MCF-7, KIP-cyclin D1-Cdk4 assembly occurred at 6-9 hours, while the loss of p27 and p21 from cyclin E complexes was notable only somewhat later, after the time of peak sequestration of these KIPs in cyclin D1 complexes. Although induced overexpression of cyclin D1 can abrogate antiestrogen arrest ^{244,245}, the physiologic upregulation of cyclin D1 stimulated by estradiol in MCF-7 may be insufficient, on its own, to mediate the shifts of the KIPs out of Cdk2.

The ubiquitin-mediated degradation of p27 ^{43,253,254} requires its phosphorylation on Thr187 ^{40,41,247}. Degradation of p21 is also proteasome dependent, but may differ importantly from that of p27 ²⁴⁶. While cyclin E-Cdk2 acts in vitro and in vivo to phosphorylate p27 on Thr187, other kinases may act on p27 prior to its degradation. The transition of p21 and p27 from potent inhibitors of cyclin E-Cdk2 in G0 to cyclin D-dependent kinase assembly factors may require phosphorylation early in G1, prior to cyclin E-Cdk2 activation. We have observed an increase in p27 phosphorylation prior to the reduction in its steady state levels in estradiol stimulated MCF-7 (S. Cariou and J. Slingerland, unpublished results). MAPK activation has been implicated in p27 degradation ^{130,255}. It will be of interest to determine if the estradiol-dependent activation of MAPK ^{117,227} or other mitogenic kinase pathways regulate the transition

of p21 and p27 from high affinity inhibitors of cyclin E-Cdk2 to activators of cyclin D1-Cdk assembly.

The approximation that over 4 million women with breast cancer are on Tamoxifen worldwide is a minimal estimate (^{214,215}and V.C. Jordan personal communication). An increasing body of in vitro data, and meta-analysis of large patient cohorts have confirmed the requirement for ER expression for the therapeutic efficacy of Tamoxifen ^{214,215,256}. The present study suggests that in addition to the ER, a breast cancer cell must express functional p21 and p27 for Tamoxifen or Faslodex (ICI 182780) to mediate cytostatic effects. These observations raise the hypothesis that deregulation and loss of these KIPs may underlie the clinical phenomena of hormone independence and antiestrogen resistance in breast cancer.

Section III

Constitutive MEK/MAPK Activation Leads to p27^{Kip1} Deregulation and Antiestrogen Resistance in Human Breast Cancer Cells

A version of this section is published in *The Journal of Biological Chemistry* with the DOD awardee as first author and is appended in Appendix 2

(data from fig 1 and 3 were presented at the time of last year's report. Data from Fig 2, 4 and 5 have been conducted since the time of last year's report)

Summary of Section III

As I reported last year in the Annual Report, antiestrogens require action of the cell cycle inhibitor p27^{Kip1} to mediate G1 arrest in estrogen receptor positive (ER+) breast cancer cells. I report here in Section III that constitutive activation of the mitogen activated protein kinase (MAPK) pathway alters p27 phosphorylation, reduces p27 protein levels, reduces the Cdk2 inhibitory activity of the remaining p27, and contributes to antiestrogen resistance. In two antiestrogen resistant cell lines that showed increased MAPK activation, inhibition of the MAPK kinase (MEK) by addition of U0126 changed p27 phosphorylation and restored p27 inhibitory function and sensitivity to antiestrogens. Using antisense p27 oligonucleotides, we demonstrated that this restoration of antiestrogen-mediated cell cycle arrest required p27 function. These data suggest that oncogene-mediated MAPK activation, frequently observed in human breast cancers, contributes to antiestrogen resistance through p27 deregulation.

SPECIFIC INTRODUCTION TO SECTION III

As reviewed in the Introduction of Section I, p27^{kip1} is a member of the KIP (kinase inhibitory protein) family of Cdk inhibitors that regulate the cyclin/Cdk complexes governing cell cycle transitions¹⁴. The importance of p27 as a G1-to-S phase regulator is highlighted by the finding that antisense-mediated inhibition of p27 expression is sufficient to induce cell cycle entry in quiescent fibroblasts³⁵ and in steroid-depleted breast cancer cells (presented in Section II)³⁶. p27 protein levels are high in G0 and early G1 during which time p27 binds tightly and inhibits cyclin E1-Cdk2. p27 translation rates decrease and its proteolysis increases during G1-to-S phase progression, leading to p27 protein loss as cells enter S phase^{253,258,259}. p27 proteolysis is regulated by phosphorylation of p27 on threonine 187 (T187) by cyclin E1-Cdk2^{40,41}. While mutations or deletions in the p27 gene are uncommon^{260,261}, p27 degradation is increased in many cancers, including breast cancer^{232,262}.

An increasing body of data suggests that p27 is regulated by mitogenic signal transduction pathways, including Ras-dependent activation of the mitogen-activated protein kinase (MAPK) pathway^{63,130,209,263,264}. Many mitogens increase the cellular levels of GTP-bound Ras, leading to activation of the downstream target, Raf-1. The Raf-1 kinase can phosphorylate and activate the dual specificity kinases MEK1 and MEK2, which in turn activate MAPK (also known as p42^{ERK2} and p44^{ERK1}). Once activated, MAPK can phosphorylate several nuclear transcription factors including Myc, Elk and Rsk (for review, see²⁶⁵). p27 itself has several MAPK consensus sites and MAPK can phosphorylate p27 *in vitro*²⁶³ and reduce the ability of recombinant p27 to bind and inhibit Cdk2 *in vitro*¹³⁰. While constitutive activation of Ras-MAPK can reduce p27 inhibitory function in immortal and cancer-derived lines, it is not clear whether MAPK directly regulates p27 during cell cycle progression in normal cell types.

Studies of p27 regulation by the Ras-MAPK pathway were initially carried out in fibroblasts ^{67,68,130}. In NIH3T3 fibroblasts, Ras signaling is required for the downregulation of p27 as cells approach the G1-to-S phase transition ^{68,209}. Introduction of a dominant negative *ras* mutant prevented the loss of p27 in response to serum and inhibited S phase entry. Others have reported that Ras-MAPK activation reduces the ability of p27 to inhibit Cdk2 through sequestration of p27 into cyclin D1-Cdk4 complexes, rather than by promoting p27 protein loss

63

Constitutive activation of the MAPK cascade may contribute to malignant progression of many human cancers ²⁶⁶. Although the causes of MAPK activation differ among tumors, in many cancers constitutive signaling from cell surface tyrosine kinase receptors contributes to activation of the Ras-Raf-1-MEK-MAPK pathway. For example, the epidermal growth factor receptor (EGFR) and HER-2/c-ErbB-2, both of which activate the Ras-MAPK pathway, are overexpressed in up to 20 % and 30 % of breast cancers, respectively. Overexpression of these receptors has been associated with antiestrogen resistance and poor prognosis in primary breast cancers ²⁶⁷⁻²⁷². Tissue culture models suggest that elevated MAPK activity may contribute to estrogen independent growth of breast cancer cells ²⁷³⁻²⁷⁵.

Antiestrogen drugs, such as Tamoxifen, are effective in the treatment and prevention of breast cancer ^{276,277}. However, only two-thirds of estrogen receptor (ER) positive breast cancers respond initially to antiestrogen therapy and even sensitive tumors invariably acquire antiestrogen resistance ²¹⁵. In most cases, acquired resistance is not due to a loss or mutation of the ER ^{221,278}. Numerous mechanisms have been proposed to explain the phenomenon of Tamoxifen resistant ER positive breast cancer, including altered drug metabolism ²⁷⁹, altered binding of co-activator and co-repressor molecules to the antiestrogen-ER complex ³³, and

altered signal transduction pathways that modulate ER activity¹²³ or regulate the cell cycle machinery³⁶.

The cell cycle inhibitor, p27^{Kip1}, is an essential mediator of cell cycle arrest by Tamoxifen and other antiestrogenic drugs. I demonstrated in Section II that antisense-mediated downregulation of p27^{Kip1} abrogated antiestrogen induced cell cycle arrest in the ER positive MCF-7 breast cancer line³⁶. p27 protein levels are frequently reduced in primary breast cancers compared to the normal breast epithelium and low p27 protein levels are associated with poor prognosis and hormone independence^{128,231,232}. These observations stimulated the present study to investigate the relationships between Ras-MAPK pathway activation, antiestrogen resistance and p27 function. Our data indicate that constitutive MEK activation alters p27 phosphorylation, reduces p27 inhibitory activity, and contributes to antiestrogen resistance in breast cancer.

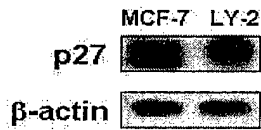
RESULTS

Is p27 altered in antiestrogen resistant cell lines?

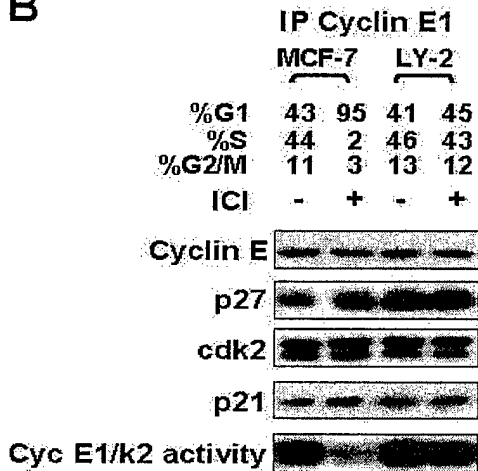
As reported in last year's summary, we compared p27 levels in antiestrogen sensitive MCF-7²³⁹ and the resistant MCF-7 derivative, LY-2²⁸⁰. The loss of antiestrogen responsiveness in LY-2 was not due to a loss of p27 protein (Fig. 1A). The association of p27 with cyclin E1-Cdk2 was assayed in asynchronously growing and antiestrogen treated MCF-7 and LY-2 cells (Fig. 1B). Asynchronously proliferating LY-2 and MCF-7 cells had similar cell cycle profiles (Fig. 1B). When equal amounts of cyclin E1 were immunoprecipitated, the amount of p27 bound to cyclin E1-Cdk2 in asynchronously growing LY-2 was nearly four-fold higher than that in asynchronous MCF-7. There was no compensatory decrease in p21 binding to cyclin E1 in proliferating LY-2. Levels of cyclin E1 bound p21 were similar in proliferating MCF-7 and LY-2. Cyclin E1 bound Cdk2 levels were similar in the two lines and were not affected by

Fig1

A



B



C

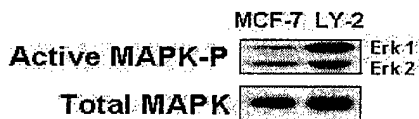


Figure 1. Antiestrogen resistant LY-2 show altered p27 regulation. (a) Asynchronously growing MCF-7 and LY-2 cell lysates were analyzed by western blotting using the antibodies indicated. (b) Cyclin E1 immunoprecipitates (IP) from asynchronously proliferating and ICI 182780 (ICI)-treated cells were resolved and assayed for associated p27 and Cdk2 by immunoblotting or analyzed for associated histone H1 kinase activity as described Experimental Procedures. The cell cycle profiles from flow cytometric analysis are shown. (c) Lysates from asynchronously proliferating cells were analyzed for levels of total and active (phospho) MAPK.

antiestrogens. Despite the increased p27 bound to cyclin E1-Cdk2 in proliferating LY-2 cells, the histone H1 activity of these complexes was not reduced compared to cyclin E1-Cdk2 from asynchronous MCF-7 (Fig. 1B). Antiestrogen treatment of MCF-7 with either ICI 182780 (ICI) or 4-OH-TAM (data shown here for ICI) caused a three to five-fold increase in p27 binding to cyclin E1-Cdk2, a three-fold increase in cyclin E1-bound p21, inhibition of this kinase, and G1 cell cycle arrest. Antiestrogen treatment of LY-2 caused a minimal increase in p21 binding, no change in the amount of p27 bound to cyclin E1-Cdk2, no significant inhibition of cyclin E1-Cdk2 activity, and no change in the cell cycle profile (Fig. 1B). These data suggested a functional alteration of p27 in LY-2 cells.

What kinases could be responsible for altered p27 in LY-2?

Since MAPK had been shown to alter p27 function in fibroblasts^{67,68,130}, we assayed MAPK activity by Western blotting using phosphospecific antibodies that detect activated MAPK. Although total MAPK protein levels were similar in LY-2 and MCF-7 cells, the levels of phosphorylated MAPK (both p42 ERK2 and p44 ERK1) were elevated nearly eight-fold in LY-2 (Fig. 1C). We also observed elevated MAPK activity in two other MCF-7 derived antiestrogen-resistant cell lines, LCC2 and MCF-7/HER-2-18. These data were reported in last year's annual report.

Does MEK overexpression confer antiestrogen resistance?

To determine whether the increased MAPK activity observed in the antiestrogen resistant lines was causally linked to the antiestrogen resistant phenotype, we transfected MCF-7 cells with activated MEK1 (MEK^{EE}) or an active ERK2 allele (ERK2-MEK), and assayed stable transfected cell lines for antiestrogen sensitivity. Transfectants showed MAPK activation compared to parental cells and empty vector controls (representative data shown for MEK^{EE} transfectants, labeled M1 and M2 in Fig. 2A). These set of experiments have been conducted

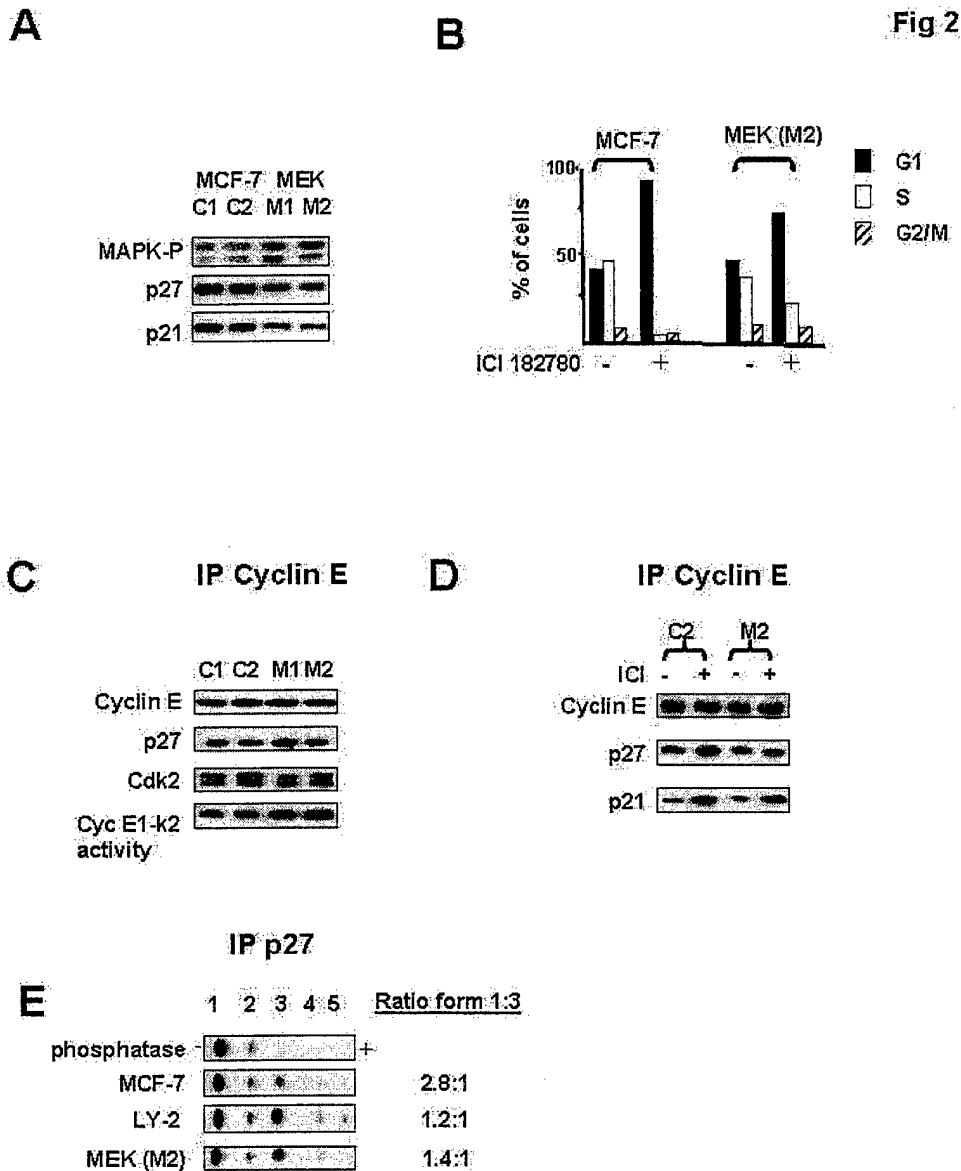


Figure 2. MAPK activation contributes to p27 deregulation and antiestrogen resistance. (a) The levels of active phospho-MAPK, p27 and p21 were analyzed in two control cell lines transfected with empty vector (C1,C2) and in two MEK overexpressing MCF-7 clones (M1, M2). (b) The cell cycle profiles of asynchronously proliferating and antiestrogen-treated MEK transfectants were compared to empty vector controls. (c) Cyclin E1 bound p27 and Cdk2, and cyclin E1-associated kinase activities were assayed as in Fig. 1B. (d) Cyclin E1-bound p21 and p27 were assayed before (-) and after (+) ICI treatment. (e) p27 immunoprecipitates from asynchronously proliferating MCF-7, LY-2 and MEK^{EE} transfectant, M2, were analyzed by 2D-IEF. The 2D-IEF of phosphatase-treated p27 from MCF-7 cells is shown in the upper panel. The different p27 isoforms were quantitated by densitometry.

since last year's annual summary report to the DOD. Of note, both p21 and p27 levels were reduced in MEK^{EE} transfectants (Fig. 2A). The level of the ER protein was not affected by the degree of MAPK activation achieved in these cells (data not shown). Asynchronously growing MAPK-activated lines and empty vector controls showed similar cell cycle profiles (Fig. 2B). Lines with constitutive MAPK activation showed partial resistance to 4-OH-TAM or ICI compared to the parental or vector alone controls (Fig. 2B).

What cell cycle changes result from MEK activation?

Levels of cyclin E1 associated p21 and p27 were assayed in asynchronous MEK^{EE} transfectant lines and in the empty vector controls (Fig. 2C). Although densitometric analysis showed that total p27 levels in asynchronously growing MEK^{EE} transfectants were reduced by up to three-fold compared to controls, the amount of p27 detected in cyclin E1-Cdk2 complexes was not reduced (Fig. 2C and D). Despite the similar amounts of both p27 and p21 bound to cyclin E1 in proliferating MEK^{EE} and control lines, equal amounts of cyclin E1-Cdk2 showed approximately two-fold higher kinase activity in MEK^{EE} transfectants compared to empty vector controls (Fig. 2C and D). There was no change in Cdk2-binding to cyclin E1 and the subtle increase in the proportion of the faster mobility, CAK-activated Cdk2 bound to cyclin E1 would not suffice to mediate the two-fold increase in cyclin E1-Cdk2 activity in the MEK^{EE} lines (Fig. 2C). MCF-7 lines with constitutive MEK1 activation showed no increase in p27 binding to cyclin E1 following antiestrogens compared to that in parental MCF-7 or in the empty vector controls (representative data in Fig. 2E). The modest increase of p21 binding to cyclin E1-Cdk2 may mediate the partial cell cycle inhibition after antiestrogen treatment of the M2 clone. Earlier work has established that increased KIP binding to cyclin E1-Cdk2 in MCF-7 is essential for G1 arrest by antiestrogens^{36,283}. Thus, MAPK activation via MEK^{EE} or ERK2-MEK transfection

may contribute to antiestrogen resistance, at least in part, by impairing the antiestrogen-mediated increase in p27 binding to cyclin E1-Cdk2.

Does MEK activation alter p27 phosphorylation?

The MAPK activated transfectants and LY-2 cells show a number of similarities. Both showed more abundant p27 binding to cyclin E1-Cdk2 in asynchronously proliferating cells than would have been predicted from the respective total cellular abundance of p27, and antiestrogens failed to cause an accumulation of p27 in cyclin E1-Cdk2 complexes. We postulated that differences in p27 phosphorylation may be associated with these differences in p27 function. Under most one-dimensional SDS-PAGE conditions, p27 does not show reproducible differences in gel mobility. Two-dimensional isoelectric focusing (2D-IEF) allowed for resolution of different p27 phospho-isoforms that are not apparent on single dimension gel electrophoresis. 2D-IEF showed a reproducible difference between the phosphorylation profile of p27 in the antiestrogen sensitive and resistant lines. 2D-IEF of p27, using an amphoteric carrier with a non-linear pH range of 3 to 10, showed five p27 isoforms present in all three lines (labeled 1-5 in Fig. 2E). Form 1 migrates at the predicted isoelectric focusing point for p27 (pH= 6.54). Phosphatase treatment of the p27 immunoprecipitates confirmed that most of these different isoforms represent different phosphoforms of p27 (Fig. 2E). The minor amount of form 2 remaining after phosphatase treatment may reflect incomplete dephosphorylation. Alternatively, this may represent an hypophosphorylated form of p27 in which post-translational modification (e.g. myristylation) confers a more negative charge. When cells were ^{32}P -orthophosphate labeled prior to p27 immunoprecipitation and resolution by 2D-IEF, isoforms 1 and 2 were not radiolabeled, confirming their hypophosphorylated state (data not shown).

The relative abundance of the different isoforms of p27 differed between MCF-7 and the MAPK activated lines, LY-2 and MCF-7/MEK^{EE}. In MCF-7, most of the p27 focused as isoform

1, with a lesser amount as isoform 3 and the ratio of these two forms (form 1:form 3) quantitated by densitometry was 3:1. The 2D-IEF patterns of p27 from LY-2 and the MEK transfectants were similar and both differed from that seen in MCF-7. In these antiestrogen resistant lines, form 3 showed greater relative abundance and the ratios of form 1 to form 3 were similar (approximately 1:1 in MEK^{EE} clone M2 and LY-2, Fig. 2E). These observations support the notion that MEK/MAPK activation modulates p27 phosphorylation in these resistant cell lines. Although we had reported a difference in the 2DIEF pattern of p27 from LY-2 compared to MCF-7 in last year's annual summary, the results obtained following 2DIEF in the MEK transfectants have been obtained since last year's report.

Does MEK inhibition restore sensitivity to antiestrogens in resistant cells?

As we reported last year, the combination of MEK inhibition and antiestrogen treatment caused resistant cells to commit to G1 arrest. Treatment of the LY-2 line with 0.1 μ M of the MEK inhibitor, U0126, caused a 2.5 fold reduction of phospho-MAPK levels without affecting the total MAPK protein levels (Fig. 3A). Although this low dose of U0126 alone did not affect the cell cycle profile of the LY-2 cells, treatment with the combination of 0.1 μ M U0126 and either 1 μ M 4-OH-TAM or 10 nM ICI led to a G1 arrest (data shown for ICI treatment, Fig. 3B and C). MEK inhibition by 0.1 μ M U0126 also restored 4-OH-TAM or ICI-mediated G1 arrest in the antiestrogen resistant HER-2/ErbB-2 overexpressing line, MCF-7/HER-2-18 (Fig. 3B). The G1 arrest following the combination of MEK inhibition and antiestrogen treatment in LY-2 (U+ ICI) was accompanied by a five-fold increase in the binding of p27 to cyclin E1-Cdk2 complexes (Fig. 3C) and inhibition of cyclin E1-Cdk2 activity (data not shown). p21 binding to cyclin E1-Cdk2 was also modestly increased and the proportion of CAK-phosphorylated Cdk2 (faster mobility) bound to cyclin E1 was modestly reduced by the combined ICI 182780 and U0126 treatment.

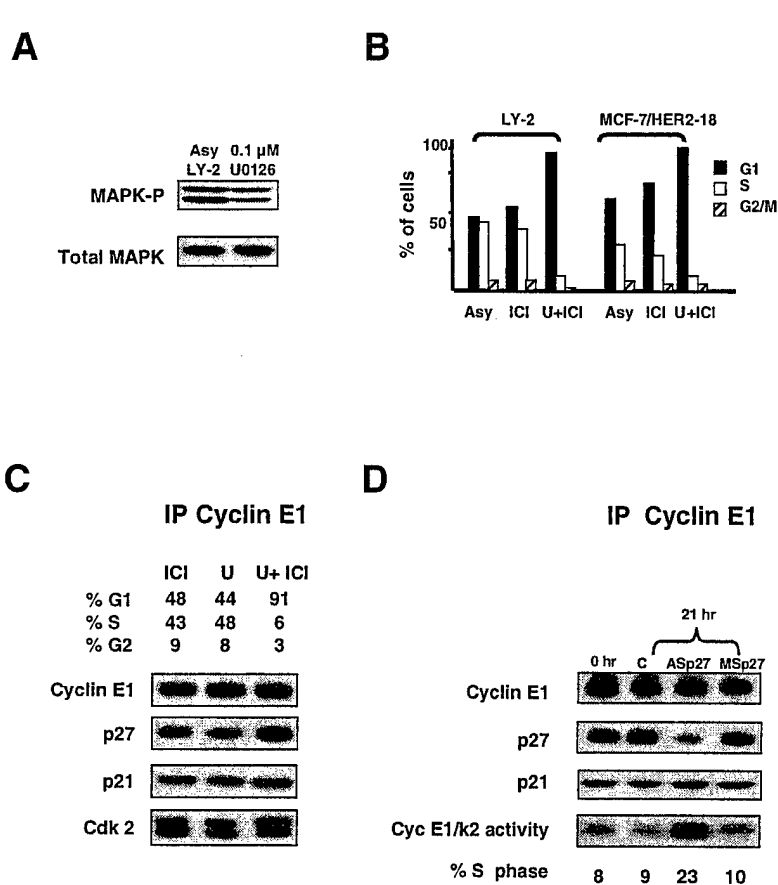


Figure 3. Inhibition of MAPK restores sensitivity to antiestrogens in LY-2. (a) Asynchronously growing LY-2 or LY-2 cells treated with 0.1 μ M U0126 were assayed for levels of total and active MAPK by immunoblotting. (b) The cell cycle profile of LY-2 and MCF-7/HER-2 cells were measured in asynchronously proliferating, ICI treated and 0.1 μ M U0126 + ICI (U+ICI) treated cells. (c) LY-2 cells treated for 48 hours with ICI, U0126 (U) or the combination of U0126 + ICI (U+ICI) and cell cycle profiles assayed by flow cytometry. Cyclin E1 immunoprecipitates were resolved and analyzed by immunoblotting with the indicated antibodies. (d) LY-2 cells treated with the combination of U0126 + ICI were lysed before (0 hr) or 21 hours after transfection with lipid only (control group, C), ASp27 oligonucleotides (ASp27) or MSp27 oligonucleotides (MSp27). Cyclin E1 immunoprecipitates were resolved and associated proteins detected by immunoblotting. The %S phase cells and cyclin E1-associated kinase activities in each treatment group are shown.

Is the the arrest of LY-2 by MEK inhibition and antiestrogens p27-dependent?

The increase in p27 association with cyclin E1-Cdk2 in LY-2 cells treated by the combination of 0.1 μ M U0126 and 10 nM ICI was similar to that seen following antiestrogen treatment in the sensitive MCF-7 line (Fig. 2E). We postulated that MAPK inhibition in LY-2 enhanced the Cdk2 inhibitory function of p27 to facilitate cell cycle arrest by antiestrogens. If this were the case, then antisense-mediated inhibition of p27 expression in the U0126/ICI treated cells should abrogate this drug-mediated arrest. U0126/ICI arrested LY-2 cells were transfected with antisense p27 (ASp27) oligonucleotides, mismatch control oligonucleotides (MSp27), or mock transfected with lipid only (control, C) and cells were recovered for flow cytometry and protein analysis at 21 hours following completion of ASp27 transfection. The inhibition of p27 expression in ASp27 treated cells lead to cell-cycle re-entry with approximately 23 % cells in S phase at 21 hours, in contrast to 8% and 9% of cells in S phase following lipid only (control, C), or mismatch (MSp27) transfection (Fig. 3D). The ASp27-mediated cell cycle re-entry was associated with loss of cyclin E1-bound p27 and cyclin E1-associated kinase activation (Fig. 3D). Control (lipid alone) and MSp27 transfected groups showed no cyclin E1-Cdk2 activation. We also observed a similar result using the combination of 0.1 μ M U0126 and 1 μ M 4-OH-TAM (data not shown). Thus, in the LY-2 line, p27 became an essential mediator of G1 arrest by antiestrogens following partial MEK/MAPK inhibition.

Are p27 complexes different in resistant cells?

Since last year's reporting to the DOD, we uncovered an usual finding that p27 from the LY-2 line has associated cyclin E-cdk2 kinase activity. Proliferating LY-2 cells, with and without antiestrogen treatment, showed more abundant p27 association with active cyclin E1-Cdk2 than was detected in inhibited cyclin E1-Cdk2 complexes from antiestrogen-arrested MCF-7 cells (Fig. 1B). These data suggested impaired inhibitory function cyclin E1-bound of p27 in

Fig 4

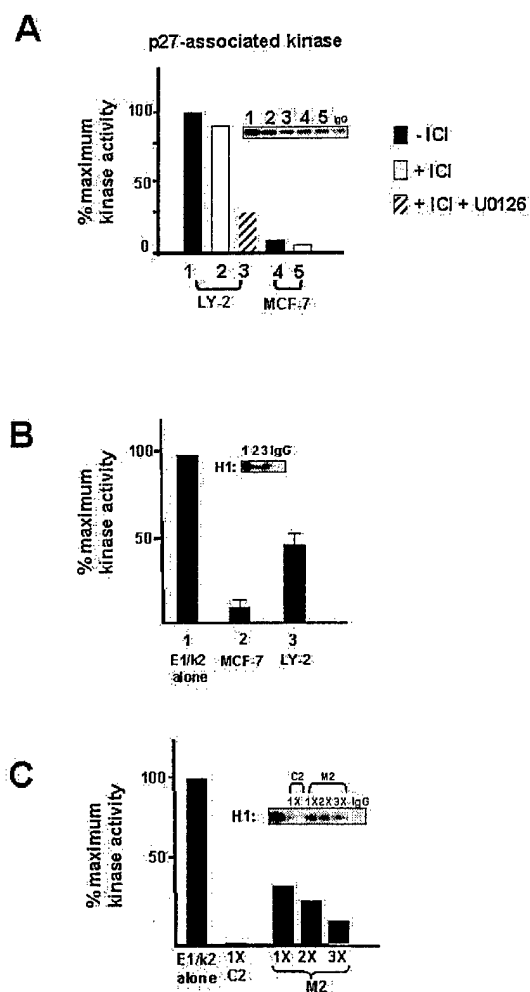


Figure 4. MEK inhibition partially restores p27 inhibitory function and changes the pattern of p27 phosphorylation. (a) Equal amounts of p27 were precipitated from asynchronous (lanes 1, 4) and ICI treated LY-2 and MCF-7 cells (lanes 2, 5), and from LY-2 cells treated with both 0.1 μ M U0126 + ICI (lane 3) and analyzed for associated histone H1 kinase activity. Radioactivity in the histone H1 substrate was quantitated by PhosphoImager and expressed as a percentage of maximum activity after subtraction of background from IgG control (lane 6) and graphed. The insert shows the autoradiogram of activity in histone H1. The data presented are the mean of repeat experiments. (b) p27 was immunoprecipitated from asynchronously proliferating MCF-7 and LY-2 lysates, boiled and equal amounts of p27 were added to recombinant cyclin E1-Cdk2. Cyclin E1 complexes were then immunoprecipitated and assayed for histone H1 (H1) kinase activity. Inhibition of cyclin E1-Cdk2 activity by added p27 is shown. Radioactivity incorporated into the histone H1 substrate is shown in the autoradiograph (see insert, upper right) and graphed as % maximum uninhibited cyclin E1-Cdk2 activity. Mouse IgG served as an immunoprecipitation control. (c) Equal amounts of p27 were immunoprecipitated from the asynchronously proliferating MCF-7/MEK^{EE} clone (M2) or from the empty vector control (C2) and cyclin E1-Cdk2 inhibitor function assayed as in (B).

LY-2 cells. p27 immunoprecipitates were tested for associated histone H1 kinase activity in LY-2 and MCF-7 cells. Cdk2 complexes, but not Cdk4 and 6 complexes can use histone H1 as substrate. Histone H1 kinase activity was detected in p27 immunoprecipitates from asynchronous and ICI-treated LY-2 cells, but was negligible in asynchronous and ICI-treated MCF-7 when background activity in non-specific antibody control immunoprecipitates was subtracted (Fig. 4A). The combination of 0.1 μ M U0126 and 10 nM ICI 182780 (U+ICI) inhibited the p27 immunoprecipitable kinase activity in LY-2 (Fig. 4A). Parallel p27 immunoprecipitates were resolved and blotted for associated Cdk2, cyclin E1 and cyclin A. The amounts of cyclin and Cdk2 bound to p27 in ICI treated LY-2 and MCF-7 were similar and there was no loss of p27 bound cyclin or Cdk2 following ICI plus U0126 treatment LY-2 cells (data not shown).

Does MEK activation make p27 a poorer cyclin E-cdk2 inhibitor?

Since the time of last year's annual reporting, we proceeded with a set of experiments which showed that p27 is a poorer inhibitory of cyclin E-cdk2 in the LY-2 line compared to MCF-7. The inhibitory activity of p27 toward recombinant cyclin E1-Cdk2 was compared in the MCF-7 and LY-2 lines (Fig. 4B) and in the MCF-7/MEK-EE transfectant, M2, and the empty vector control line, C2 (Fig. 4C). Equal amounts of p27 protein were immunoprecipitated from the indicated cell lines, boiled to release associated proteins and then heat stable p27 was tested for its ability to inhibit a fixed amount of recombinant cyclin E1-Cdk2. The cyclin E1-Cdk2 complexes were then immunoprecipitated and kinase activity assayed. The activity of the p27-treated cyclin E1-Cdk2 was expressed as a % of control, un-inhibited cyclin E1-Cdk2. p27 from the MCF-7 line had approximately four-times the inhibitory potency as p27 from the LY-2 line (Fig. 4B). Similarly, MEK overexpression in the M2 line impaired the inhibitory function of p27 (Fig. 4C). The cyclin E1-Cdk2 inhibitory activity of increasing amounts of p27 from the MEK^{EE} transfected M2 line was compared with that of p27 from the vector alone control, C2. Even a

three-fold (3X) excess of p27 in the M2 line did not achieve the same level of cyclin E1-Cdk2 inhibition shown by p27 (1X) from the control line.

Does MEK inhibition modulate p27 phosphorylation?

Our antisense experiments showed that p27 is essential for the antiestrogen arrest of LY-2 following partial MEK inhibition (Fig. 3D). Since MEK inhibition restored antiestrogen arrest, we postulated that MEK inhibition might alter p27 phosphorylation. As seen in asynchronously proliferating cells (Fig. 2E), the 2D-IEF of p27 from antiestrogen-treated MCF-7 and LY-2 cells showed five distinct p27 isoforms (labeled 1 to 5 in Fig. 5A) with isoforms 1 and 3 again being the most abundant. p27 from antiestrogen arrested MCF-7 showed a predominance of isoform 1, with the ratio of isoforms 1 to 3 being 2:1. In antiestrogen-treated LY-2 cells, form 3 was the predominant form, with the isoform 1:isoform 3 ratio at 1:2. Treatment with 0.1 μ M U0126 together with either 4-OH-TAM or ICI, changed the p27 phosphorylation profile in LY-2 cells to one that more closely resembled that in antiestrogen arrested MCF-7 cells, with the p27 isoform 1 more abundant than form 3, at a ratio of 2:1 (data shown for ICI treatment in Fig. 5A). In all cell types, ICI treatment increased the relative abundance of isoforms 4 and 5 compared to that of untreated cells.

We tested whether the changes in total cellular p27 phosphorylation were reflected by changes in the phosphorylation of cyclin E1-bound p27 (Fig. 5B). Cyclin E1-bound p27 in the ICI treated LY-2 line showed a predominance of isoform 3 (the ratio of isoform 1:isoform 3 was 1:6 by densitometry) whereas LY-2 cells arrested by the combination of MEK inhibition and antiestrogen showed a cyclin E1-associated p27 phosphorylation pattern more closely resembling that in antiestrogen arrested MCF-7 (isoforms 1:isoforms 3 ratio nearly 1:1 in both). These data suggest that the combination of MEK inhibition and antiestrogen treatment may restore the

A

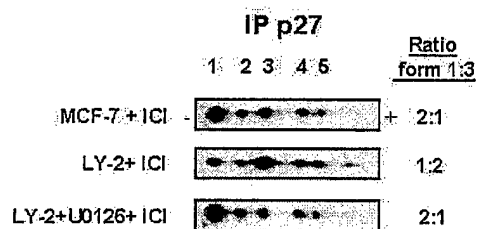


Fig 5

B

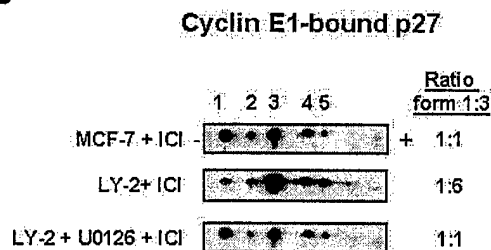


Figure 5. MEK inhibition modulates p27 phosphorylation. (a) and (b) The 2D-IEF patterns of immunoprecipitated p27 (a) or cyclin E1-bound p27 (b) were assayed in MCF-7 and LY-2 cells after 48 hours of treatment with ICI, and in LY-2 cells after 48 hours treatment with both ICI and UO126.

cyclin E1-Cdk2 inhibitory function of p27 in LY-2, at least in part, by altering p27 phosphorylation.

FURTHER DISCUSSION BY DOD AWARDEE

The key roles of p21 and p27 in antiestrogen arrest have been demonstrated in earlier studies^{36,283}. Antiestrogens increase cyclin E1-Cdk2-KIP binding, and immunodepletion of p21 and p27 from steroid-depleted or Tamoxifen arrested cells removes essentially all cellular cyclin E1-Cdk2²³⁶⁻²³⁸, suggesting that these cyclin complexes are fully saturated by p21 or p27 in arrested cells. Recent work with antisense (AS) p27 and p21 demonstrated that inhibition of expression of either KIP from antiestrogen arrested cells leads to cell cycle re-entry^{36,283}. In addition to increased KIP-Cdk binding, other cell cycle effectors contribute to G1 arrest by antiestrogens. These include reductions in c-myc, cyclin D1 and Cdc25A, increased p15 and potentially, the accumulation of Cdk2 in a non-CAK activated form^{36,236,237,245}. However, while induction of cell cycle arrest by antiestrogens has multiple effectors, the antisense studies demonstrate that KIP function is required for maintenance of arrest. Moreover, the present work indicates that deregulation of p27 inhibits antiestrogen responsiveness.

Our data suggest that constitutive MEK/MAPK activation contributes to the development of antiestrogen resistance in ER-positive breast cancer cells, at least in part, by compromising the inhibitory function of p27. We show here that a non-cytostatic and non-cytotoxic dose of the MEK inhibitor, U0126, restored sensitivity to G1 arrest by antiestrogens in the widely used LY-2 model of antiestrogen resistance. Moreover, transfection of HER-2 or MEK^{EE} into MCF-7 impaired antiestrogen responses. In antiestrogen treated LY-2 and MCF-7/MEK^{EE} transfectants, p27 failed to accumulate in cyclin E1-Cdk2 complexes and did not inhibit this kinase. MEK inhibition by U0126 in these antiestrogen resistant lines altered p27 phosphorylation, and restored the inhibitory binding of p27 to Cdk2 following antiestrogen treatment. Thus,

MEK/MAPK-dependant p27 phosphorylation events are associated with a reduced ability to inhibit Cdk2.

Through the course of selection of the antiestrogen resistant LY-2, p27 regulation has been altered such that its binding to cyclin E1-Cdk2 is increased without a commensurate reduction in cyclin E1-Cdk2 activity. In antiestrogen-mediated arrest of MCF-7, a three-fold increase in p27 binding to cyclin E1-Cdk2 is sufficient for Cdk2 inactivation and cell cycle arrest³⁶. The approximately four-fold increase in cyclin E1-bound p27 in asynchronously growing LY-2 cells relative to that in proliferating MCF-7 and the failure of antiestrogens to increase p27 binding to cyclin E1-Cdk2 in LY-2 prompted further investigation of p27 function in these lines. Indeed, both cyclin E1 and p27 immunoprecipitates contain detectable histone H1 kinase activity in LY-2 cells. While the p27 associated kinase activity could reflect dissociation of p27 from cyclin E1-Cdk2 *in vitro* following immunoprecipitation of the complexes, the increased binding of p27 to cyclin E1 without loss of kinase activity in asynchronous LY-2 suggests that some of the cyclin E1-Cdk2-p27 complexes may retain activity *in vivo*. Detection of p27-immunoprecipitable kinase activity has been reported by others^{46,184}. The elevated levels of p27 protein in the LY-2 line may reflect MAPK-independent events that have occurred throughout the course of selection of this line²⁸¹.

p27 levels were reduced in the MEK^{EE} transfectants, consistent with observation by others that Ras-MAPK contributes to p27 degradation^{68,130,284}. Despite the lower total p27 protein levels in these cells, cyclin E1-bound p27 levels were not reduced. Moreover, while cyclin E1 bound p21 and p27 levels were similar in MEK^{EE} transfectants and control lines, cyclin E1-Cdk2 activity was increased in asynchronously proliferating MEK^{EE} transfectants compared to controls. Thus, MAPK activation at the levels achieved here, may favor the association of p27 with Cdk2 in a poorly inhibitory form, such that some of the cyclin E1-Cdk2-

p27 complexes retain activity. This effect may be separable from the effect of MAPK on p27 stability. p27 forms that can bind cyclin E1-Cdk2 but fail to inhibit this kinase have been modeled previously *in vitro*⁴¹. Further evidence to support functional alteration of p27 in the LY-2 and MEK^{EE} transfected MCF-7 lines is provided by assays of p27 inhibitory function. p27 from both LY-2 and the MEK^{EE} transfected line, M2, both showed a reduced ability to inhibit recombinant cyclin E1-Cdk2 *in vitro*. As an alternate model, MEK/MAPK activation may reduce the stability with which p27 binds to cyclin E1-Cdk2 *in vitro*, allowing detection of this kinase activity in p27 IP through dissociation *in vitro*. A reduced ability to bind cyclin E1-Cdk2 could also account for the reduced inhibitory activity of heat stable p27 isolated from the resistant lines.

Signal transduction pathways have been shown to affect p27 inhibitory function^{130,285}, raising the possibility that phosphorylation events may modulate p27 function. Overexpression of the integrin-linked kinase (ILK) causes a reduction in inhibitory activity of p27 toward cyclin E1-Cdk2²⁸⁵. Here we showed that the antiestrogen ICI182780 modulates p27 phosphorylation in MCF-7 cells, increasing the relative amounts of p27 isoforms 3, 4 and 5. We also showed an association between altered p27 inhibitory function and altered phosphorylation in LY-2 and MCF-7-MEK^{EE} cells, suggesting that deregulated p27 phosphorylation may be causally linked to antiestrogen resistance. Although MAPK can phosphorylate p27 *in vitro*^{130,263,264}, it is not known at present whether direct phosphorylation of p27 by MAPK occurs *in vivo*. The effects of MAPK on the p27 phosphorylation profile may be indirect. p27 contains several potential MAPK consensus sites, including serine 10 (S10), S178, and T187. S10 has recently been shown to be a major p27 phosphorylation site in G0 arrested cells, although it may not be a physiological MAPK target site²⁶³. Since a p27 mutation converting S10 to alanine or aspartate did not affect the ability of p27 to inhibit cyclin E1-Cdk2 *in-vitro*²⁶³, the MAPK-dependent

pathway that modulates both p27 phosphorylation and its ability to inhibit Cdk2 cannot uniquely affect S10. Moreover, the phosphorylation of p27 at T187 that regulates its recognition by the F-box protein Skp-2, does not affect the Cdk2 inhibitory function of p27⁴¹. Thus, phosphorylation at sites other than S10 and T187 may be required for the MEK/MAPK dependent phosphorylation of p27 that modulates its Cdk2 inhibitory function. The identity of the different 2D-IEF phospho-isoforms of p27 observed by 2D-IEF warrants further investigation.

The causes of MAPK activation in human cancers differ among different tumors. MAPK activation is increased in up to 50% of breast cancers compared to normal breast epithelium and is associated with poor patient prognosis^{69,286 81}. HER-2/ErbB-2 overexpression, seen in up to 30 % of breast cancers is often associated with antiestrogen resistance²⁷². HER-2/ErbB-2 signaling has been shown to decrease p27 stability via MAPK activation²⁸⁴. In the HER-2 overexpressing MCF-7/HER-2-18, MEK inhibition by U0126 restored sensitivity to antiestrogens. Taken together, the present study links HER-2/ErbB-2 activation and antiestrogen resistance through MAPK-dependent alterations in p27 function.

In addition to its mechanistic relevance to breast cancer, the observed link between p27 dysfunction and MAPK activation has implications for many types of cancers. The reduced levels of p27 observed in many cancers (colon, lung, prostate, gastric) may reflect oncogenic activation of the Ras/MEK/MAPK pathway²⁶². For example, the increased p27 proteolytic activity observed in colon cancer lysates may result from oncogenic activation of *K-Ras* in these cancers²⁸⁷. There is a strong molecular rationale supporting the continued development of MEK/MAPK inhibitory drugs. A number of MEK inhibitors have shown good oral bioavailability and efficacy in preclinical trials²⁸⁸. Tumor-specific MEK inhibitors may have the potential to restore p27 protein levels and inhibitory function and thereby restrain tumor growth.

Section IV:

Work begun by the DOD awardee that may not be finished prior to funding completion

Is there an association between MAPK activation and p27 loss *in vivo* in breast tumors?

Several studies have shown that MAPK activity predicts poor prognosis in human breast cancer^{120,320}. We and others have shown that MAPK activation mediates tamoxifen resistance in breast cancer cell lines (Section III) in tissue culture or in mouse models^{119,275}. A strong association between elevated MAPK activity and reduced estrogen receptor levels has been reported in primary breast cancers¹²⁰. However, whether MAPK activation is associated with p27 loss and the development of antiestrogen resistance *in vivo* in primary human breast cancer has not been examined. Our lab and others have shown that reduced p27 protein is an independent prognostic factor for poor breast cancer outcome^{231,232,233}.

To determine whether increased MAPK activity is associated with p27 loss in breast cancer tumors, I have undertaken studies performing immunohistochemistry (IHC) using archival formalin-fixed paraffin embedded breast cancer tissue. p27 IHC is being performed as described²³¹. MAPK IHC is being performed using monoclonal phospho (active) MAPK antibodies (Cell Signaling). Following our completion of staining, the prognostic impact of p27 levels and MAPK activation will be correlated with other known clinical and histopathologic prognostic variables and with hormonal responsiveness. I hypothesize that MAPK activation will be strongly correlated with low p27 protein levels (i.e. less than 50 % of tumor nuclei staining for p27). Moreover, I predict that low p27 protein expression in breast cancers will be associated with antiestrogen treatment failure.

CHAPTER V

Non-malignant and tumor-derived cells differ in their requirement for p27^{Kip1} in TGF- β mediated G1 arrest

A version of this section V will be submitted for publication
(J. Donovan, J. Rothenstein, J. Slingerland, 2002)

(Experiments reported herein have been conducted since the time of last year's report)

Summary of Section V

Given the essential role of p27 in cell cycle arrest by antiestrogens, we investigated the requirement for p27 in cell cycle arrest by a second anti-proliferative stimuli, Transforming growth factor beta (TGF- β). I will show in this section that TGF- β induces G1 arrest in susceptible cells by multiple mechanisms that lead to inhibition of the G1 cyclin-dependent kinases (Cdks), including Cdk2, Cdk4 and Cdk6. TGF- β treatment of early passage finite-lifespan human mammary epithelial cells (HMECs) led to an accumulation of p27^{Kip1} in cyclin E1-Cdk2 complexes and kinase inhibition. The role of p27 in the G1 arrest by TGF- β was assessed by transfection of antisense p27 (ASp27) oligonucleotides into TGF- β treated HMECs. Despite a reduction in total and cyclin E-Cdk2 bound p27 following ASp27 transfection, HMECs remained arrested in the G1 phase. Maintenance of the G1 arrest was accompanied by increased association of the Cdk inhibitor p21^{WAF-1/Cip-1} and the retinoblastoma family member, p130^{Rb2}, in cyclin E1-Cdk2 complexes along with kinase inhibition. In contrast to the findings in HMECs, p27 was essential for G1 arrest by TGF- β in two tumor-derived lines. ASp27 transfection into two TGF- β responsive, cancer-derived lines, was not associated with increased compensatory binding of p21 and p130 to cyclin E1-Cdk2 and these cell lines failed to maintain G1 arrest despite the continued presence of TGF- β . Progressive cell cycle deregulation leading to impaired checkpoint controls during malignant tumor progression may alter the role of p27 from a redundant to an essential inhibitor of G1-to-S phase progression.

SPECIFIC INTRODUCTION TO SECTION V

TGF- β mediates effects on diverse cellular processes such as proliferation, growth and differentiation via cell surface receptors that in turn regulate the activity of SMAD transcription factors (reviewed in ²⁸⁹). In many normal cell types, including epithelial and melanocytic cells, TGF- β has a potent antiproliferative effect. In contrast to non-transformed cells, cancer-derived lines show reduced antiproliferative responses to TGF- β or have lost this response altogether ²⁹⁰. In most cases, the loss of TGF- β responsiveness occurs without inactivation of TGF- β receptors or the SMADs. Cell cycle deregulation is believed to contribute to the resistance of malignant cells to G1 arrest by TGF- β (reviewed in ²⁹¹).

TGF- β induces cell cycle arrest in the G1 phase via a number of pathways that lead ultimately to inhibition of the G1 cyclin-dependent kinases (Cdks). The cyclin dependent kinases (Cdks) are key mediators of progression through the cell cycle and are regulated by phosphorylation, cyclin binding, and by the binding of Cdk inhibitory proteins (reviewed in ¹⁴). During G1 to S phase progression, the D-type cyclins bind Cdk4 and Cdk6 and the E-type cyclins bind Cdk2, contributing to kinase activation and G1-to-S phase progression. The G1 phosphatase Cdc25A plays an essential role in Cdk activation by the removal of inhibitory phosphates from Cdk2 ⁶ and possibly also from Cdks 4 and Cdk6 ¹⁷⁹. Cdc25A may be transcriptionally upregulated by c-myc ⁸. Two families of Cdk inhibitory proteins oppose Cdk activation. p21^{WAF1/Cip-1}, p27^{Kip1} and p57^{Kip2} belong to the KIP (kinase inhibitory protein) family and contribute to the inhibition of cyclin E1-Cdk2 complexes in the G1 phase. p15^{INK4B},

p16^{INK4A}, p18^{INK4C}, and p19^{INK4D} belong to the INK4 (inhibitors of Cdk4 and Cdk6) family and act to inhibit Cdk4 and Cdk6 (reviewed in ¹⁴).

In several cell types, including human mammary (HMECs) and mink lung epithelial cells, TGF- β induces and stabilizes the p15 protein, which leads to its binding to, and inhibition of, Cdk4 and Cdk6 complexes ^{50,51,292}. TGF- β also causes the accumulation of p27 in cyclin E1-Cdk2 complexes leading to Cdk2 inhibition ^{171,172}. Changes in several key cell cycle regulators cooperate to induce TGF- β arrest, including downregulation of c-myc ^{172,174}, Cdc25A ¹⁷⁹ and cyclin D1 and in some cell types, upregulation of p21 ²⁹¹. Deregulation of various cell cycle targets including cyclin and Cdk overexpression, Cdk inhibitor inactivation, and Myc or Cdc25A overexpression are believed to contribute to TGF- β resistance in cancer ²⁹¹.

Although mouse embryonic fibroblasts (MEFs) from p27^{-/-} mice retain TGF- β sensitivity ³⁹, several studies have indicated an association between altered p27 regulation and the development of TGF- β resistance. Our previous work showed that the acquisition of TGF- β resistance in human mammary epithelial cells was associated with altered phosphorylation, altered Cdk inhibitory activity, and cytoplasmic mislocalization of the p27 protein ²⁹³. Although p27 mutations are rare in human tumors, increased proteasomal degradation of p27 is observed in a number of cancers, including breast, colon and prostate and the reduced levels are associated with poor patient prognosis (reviewed in ^{262,294}). Relatively little is known about the compensatory mechanisms invoked by a non-transformed cell following a reduction in p27 protein levels, although a few reports support a role for compensation by other Cdk inhibitors to maintain normal cell cycle control. For example, in serum-starved p27^{-/-} mouse embryonic fibroblasts (MEFs), the accumulation of the retinoblastoma family member, p130^{Rb2}, in cyclin E-Cdk2 complexes compensated for the p27 loss and enabled cells to undergo proliferative arrest in the G1 phase ²⁹⁵.

The present study investigated the requirement for p27^{Kip1} in maintaining G1 arrest by TGF- β in finite lifespan human mammary epithelial cells (HMECs) and in cancer-derived lines. Using antisense p27 oligonucleotides to inhibit p27 expression, we show that HMECs, but not the tumor cell lines, maintain G1 arrest following p27 downregulation via a compensatory accumulation of p21 and p130 in cyclin E-Cdk2 complexes. These data suggest that p27 plays an essential function in these malignant lines to maintain TGF- β arrest, and a redundant function in the finite-lifespan HMEC that can be compensated by other Cdk inhibitors.

RESULTS

How does TGF- β effect cell cycle in normal and malignant breast cells?

We compared the TGF- β responsiveness of human mammary epithelial cells (184 HMEC, passage 11), WM35, MCF-10A, and MCF-7 cells (Fig. 1A). 184 HMEC cells are a finite-lifespan mammary epithelial strain, MCF-10A is a spontaneously immortalized non-malignant breast epithelial cell line, the MCF-7 is a malignant breast cancer line. WM35 line is a malignant melanoma cell line that we used for comparative purposes in this study since it is a malignant although highly TGF β sensitive cell line. Cells were treated for 48 hours in the absence (-) or presence (+) of TGF- β (Fig. 1A). 184 and WM35 cells had similar sensitivity to TGF- β , undergoing G1 arrest with an approximately 80% reduction in the proportion of cells in S phase following 48 hours of TGF- β treatment (10ng/mL). The MCF-10A and MCF-7 cell lines were less sensitive than the 184 HMEC or WM35, but both underwent partial G1 arrest with 100 ng/mL TGF- β with over 50 % reduction in the proportion of cells in S phase. The data graphed represent the mean of three repeat assays.

TGF- β effects on cyclin and Cdk inhibitor levels- The levels of the relevant G1 cyclins, Cdks and Cdk inhibitors were assayed by Western analysis in 184, MCF-7 and WM35 cells in the

absence (-) or following a 48 hour exposure (+) to TGF- β (Fig 1B). Although cyclin D1 levels were similar in all three lines, cyclin E1 levels were approximately 2.5-fold greater in the untreated cancer-derived MCF-7 and WM35 cells compared to the 184 HMEC cells. Cyclin E1 and cyclin D1 levels showed no consistent alteration by TGF- β treatment in repeat assays. The levels of Cdk2, Cdk4, and Cdk6 were also unchanged by TGF- β (data not shown). The levels of p27 were higher in the cancer-derived lines, with asynchronous MCF-7 cells having approximately fifteen-times, and WM35 cells having approximately three times greater p27 levels than asynchronous 184 HMEC cells. TGF- β treatment did not alter p27 protein levels in the HMEC, but levels rose by approximately 1.5-fold in the MCF-7 line and three-fold in the WM35. Total p21 levels were similar in all three lines. p21 levels were unchanged in the 184 and MCF-7 cells following TGF- β . The WM35 cells showed a transient increase in p21 levels at 18-30 hours of TGF- β treatment followed by a return to similar levels as in the asynchronous population by 48 hours. p15 levels were much higher in the 184 HMEC compared to the MCF-7 line; WM35 are p15 null due to a deletion of the gene ¹⁸⁴. TGF- β treatment of 184 HMEC cells led to a three-fold increase in p15 levels following TGF- β treatment. In MCF-7 cells, p15 could not be detected in the short exposure times (3-5 minute) that were used to detect p15 from the 184 HMEC. However, a longer exposure of the film (1 hr) showed that p15 levels increased by 1.5 to 2-fold in TGF- β treated MCF-7 cells (Fig. 1C).

Given that the p130^{Rb2} protein, like p21^{WAF-1/Cip-1} and p27^{Kip1}, has a Cdk inhibitory domain and can also accumulate in and inhibit Cdk complexes ²⁹⁵, p130 protein levels were assayed in asynchronously proliferating and TGF- β treated cells by Western analysis. p130 levels were much lower in HMEC cells compared to the cancer-derived lines (Fig. 1B). TGF- β

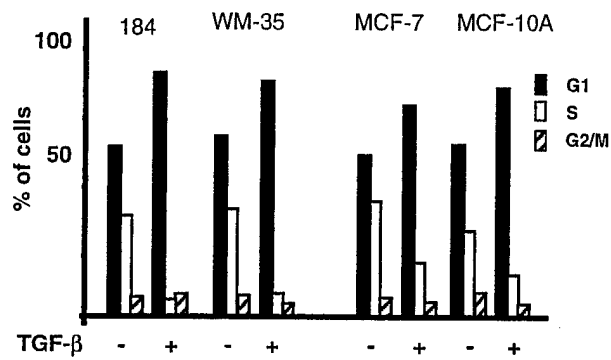
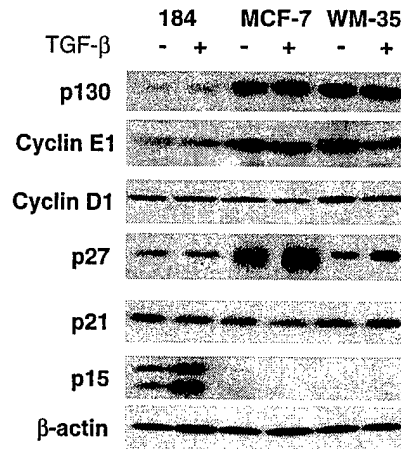
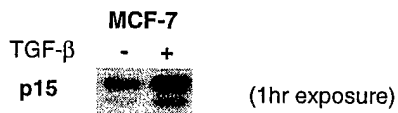
A**B****C**

Figure.1. Effects of TGF- β on the cell cycle profile and G1 regulatory proteins. *A*, Flow cytometric analysis of asynchronously proliferating (-) and 48 hr TGF- β (+) treated 184, WM35, MCF-10A and MCF-7 cells. *B*, Cell lysates from the treatment groups in (*A*) were analyzed by Western blotting using the indicated antibodies. *C*, 1 hour exposure of the film shown in *B* of p15 protein in MCF-7 in the absence (-) and following (+) 48 hr TGF- β treatment. 184 and WM35 were treated with 10 ng/mL TGF- β ; MCF-10A and MCF-7 with 100ng/mL TGF- β .

modestly increased p130 levels (less than 1.5- fold) in HMEC, but did not alter levels in the tumor-derived lines. Equal loading was verified by β -actin.

How does TGF- β effect cyclin-Cdk composition and activities?

The levels of p21, p27, p130 and Cdk2 in cyclin E1 complexes were assayed following immunoprecipitation of equivalent levels of cyclin E1 from asynchronously proliferating and TGF- β treated 184, MCF-7 and WM35 cells (Fig 2A). Cyclin E1-bound Cdk2 levels were similar and were not altered by TGF- β in repeat assays in all three lines. Cyclin E1-bound p21 levels were unaltered in 184 HMEC cells following TGF- β treatment, but TGF- β treatment of MCF-7 and WM35 cells led to a modest increase (1.5 to 2 fold) in p21 binding to cyclin E1. p27 increased in cyclin E1 complexes in all three cell types following TGF- β treatment (Fig 2B). Paradoxically, asynchronously proliferating cancer-derived lines showed a greater amount of cyclin E-bound p27 than did HMECs. Cyclin E1-bound p27 levels were approximately 8-15 times higher in proliferating MCF-7 and WM35 than in 184 cells. Cyclin E1-bound p21 was also two fold higher in the cancer derived lines than in 184 HMEC. p130 was detected in cyclin E1 complexes in both asynchronously proliferating and TGF- β treated cells (Fig 2A). Although the total p130 levels were much lower in 184 HMEC cells compared to the cancer-derived lines (see again Fig 1B), the levels of cyclin E1-bound p130 were approximately 5 to 10 fold higher in 184 HMEC cells than in the cancer derived lines. p130 binding to cyclin E1-Cdk2 was only modestly increased by TGF- β treatment in all three cell types.

The histone H1 kinase activity of cyclin E1-complexes shown in Fig 2A was assayed as described in the Experimental Procedures (Fig 2C). Although equal amounts of cyclin E1 were precipitated, cyclin E1-associated kinase activities in the cancer-derived lines were four-to-five times greater than that in the asynchronously proliferating 184 HMEC. TGF- β treatment of the

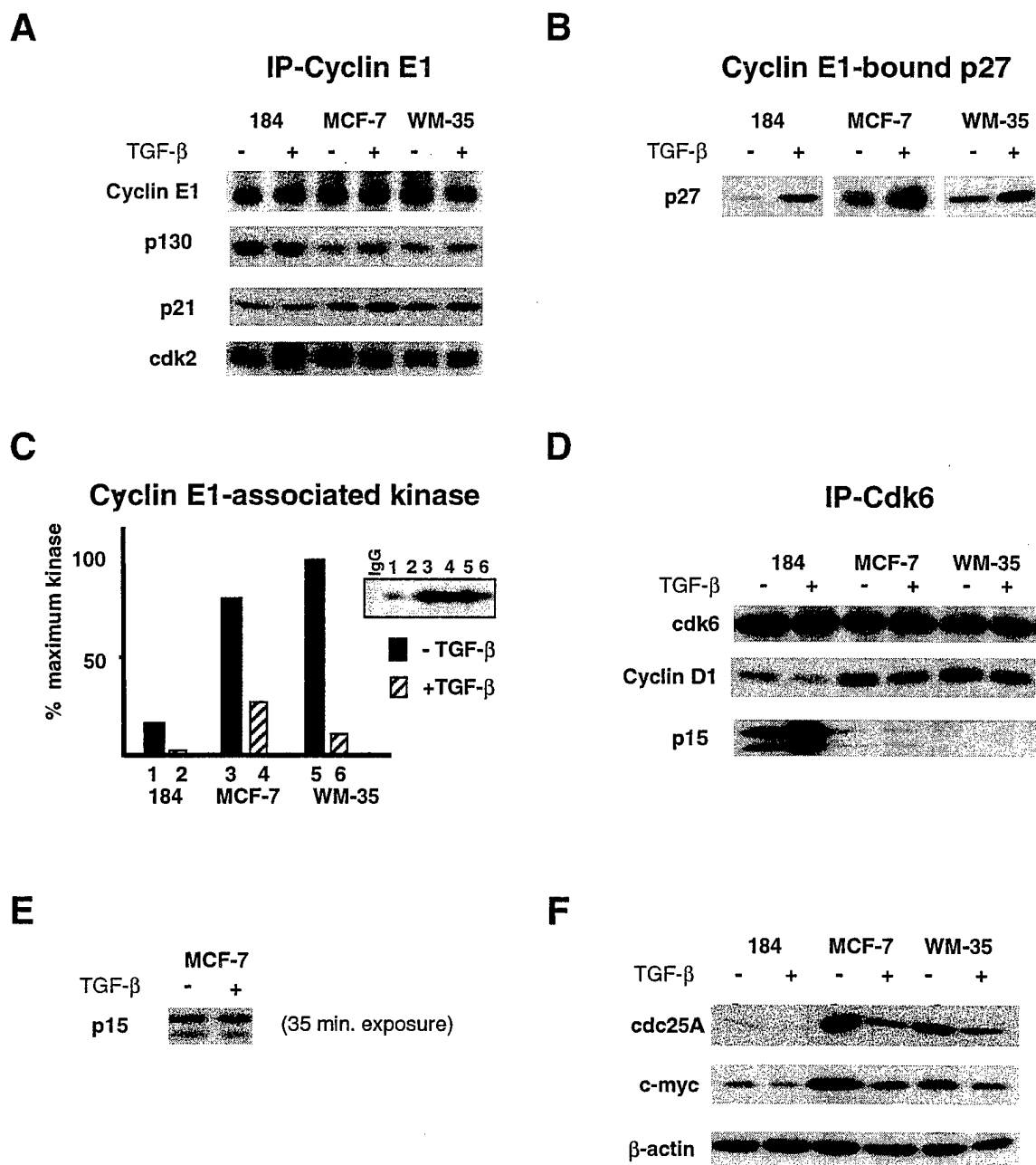


Figure 2. G1 Cdk complexes are regulated differently in normal and cancer-derived lines. *A*, Cyclin E1-immunoprecipitates from asynchronously proliferating and TGF- β treated 184, MCF-7 and WM35 cells were resolved and assayed for associated p21, p130 and Cdk2, or *B*, associated p27. *C*, Cyclin E1 immunoprecipitates were also analyzed for associated histone H1 kinase activity as described in "Experimental Procedures." *D*, Cdk6 immunoprecipitates from asynchronously proliferating of TGF- β treated cells were resolved and assayed for associated cyclin D1 and p15. *E*, Cdk-6 associated p15 following 35-minute film exposure. *F*, Western analysis of cMyc and Cdc25A levels in asynchronously proliferating and TGF- β treated cells.

184 HMEC led to a nearly complete (>95%) inhibition of cyclin E1-associated kinase activity. WM35 cells also showed 90 % reduction in kinase activity associated with the G1 arrest. Cyclin E1-associated kinase activity in the MCF-7 cells was reduced by TGF- β by approximately 60 %. The data graphed represents the average of repeat assays. Representative histone H1 kinase autoradiography is shown in the inset.

Loss of p15 upregulation by TGF- β in MCF-7 and WM35. We assayed the levels p15 and cyclin D1 present in Cdk6 complexes from asynchronously proliferating and TGF- β treated cells (Fig 2D). Despite similar total cyclin D1 and Cdk6 levels, there was more cyclin D1 bound to Cdk6 in asynchronously proliferating MCF-7 and WM35 than in 184 HMEC (see Fig 1B and 2D). TGF- β caused a two to three-fold reduction in the levels of Cdk6-bound cyclin D1 in 184 cells, while cyclin D1-Cdk6 association was not reduced by TGF- β in the MCF-7 and WM35 lines. Cdk6 bound p15 was significantly higher in asynchronously proliferating 184 than MCF-7 cells. The level of p15 bound to Cdk6 increased approximately 5-fold following TGF- β arrest of the 184 HMEC. In contrast, levels of p15 were significantly reduced and Cdk6-bound p15 levels did not increase following TGF- β treatment of MCF-7, even on longer exposure of the Cdk6-associated p15 blot (shown in Fig 2E). WM35 cells lack Cdk6-bound p15 due to a bi-allelic loss of the *p15* gene¹⁸⁴.

Increased c-myc and Cdc25A levels in cancer derived lines. The cancer-derived lines in our study showed a number of differences in the regulation of p27, cyclin D1, cyclin E1, and p15 compared to the 184 HMEC. The levels of cyclin E1-Cdk2 bound p27 were increased despite elevated cyclin E1-associated kinase activities in both asynchronously proliferating and TGF- β treated MCF-7 and WM35 lines. We observed increased cyclin D1 bound to Cdk6 complexes and a failure to accumulate p15 in Cdk6 complexes following TGF- β treatment of the cancer-derived lines. These observations prompted us to assay the levels of c-myc, since c-myc

has been shown to interfere with p27 function at many levels and to repress p15 induction^{180,299-301}. c-myc may also transactivate the *Cdc25A* gene⁸, whose product is an important activator of Cdk2 and whose downregulation plays an important role in G1 arrest by TGF- β ¹⁷⁹.

The levels of c-myc and Cdc25A proteins were assayed in the cancer-derived MCF-7 and WM35 cell lines and in the 184 HMEC (Fig 2F). c-myc levels were five to ten-fold greater in the cancer-derived MCF-7 and WM35 lines; Cdc25A levels were approximately fifteen to twenty-fold higher. TGF- β treatment of the cancer-derived lines failed to downregulate c-myc and Cdc25A proteins to the same extent as was observed in the finite lifespan 184 HMEC, with five to ten-fold higher levels of c-myc and Cdc25A remaining in the TGF- β treated cancer lines than in the arrested 184 HMEC.

Do normal and malignant breast cells differ in their ability to maintain G1 arrest following loss of p27?

As mentioned in the Introduction of this Report (Section I) p27 was discovered as a mediator of cyclin E-Cdk2 inhibition and G1 arrest by TGF- β ^{171,172}. However, in different cell types other changes in G1 regulators appear to contribute to TGF- β mediated arrest (reviewed in²⁹¹). To specifically address the requirement for p27 in TGF- β -mediated G1 arrest, we tested whether the antisense-mediated inhibition of p27 expression would abrogate TGF- β arrest in 184 HMEC, WM35, MCF-7 cells or MCF-10A cells. Cells were treated with TGF- β for 36 hours followed by a six-hour transfection with antisense-p27 (ASp27) or mismatch (MSp27) oligonucleotides or lipid alone (C) as controls. Fresh media containing TGF- β was added back following transfection. Cell cycle and protein analysis were performed immediately after the transfection and at 24 hours. p27 protein levels were reduced by three-to-five fold following ASp27 transfection and levels remained low at 24 hours post-transfection (Fig 3A). p27 levels in the control and mismatch oligonucleotide-transfected groups were similar. The transfection did

not alter protein levels of other G1 regulators examined including p21, p130, cyclin E1, cyclin D1 and Cdk2 and Cdk6 (data not shown).

ASp27 caused TGF- β arrested MCF-7 and WM35 to re-enter the cell cycle but had no such effect on arrested 184HMEC or MCF-10A cells. Flow cytometric analysis at 24 hours showed that ASp27 transfection led to a decrease in the proportion of cells in G1 and an increase in the proportion in S phase in the tumor-derived WM35 and MCF-7 lines (Fig 3B). Approximately 25-30 % of these cells were in S phase at 24 hrs post-transfection compared to only 9-15 % for the lipid and mismatch controls. Equal amounts of cyclin E1 were immunoprecipitated from ASp27 treated and from MSp27 or lipid controls, and histone H1 kinase activities were assayed. Reactivation of cyclin E1-dependent kinase accompanied cell cycle re-entry following antisense mediated inhibition of p27 expression in TGF- β treated MCF-7 and WM35 (shown for WM35 in Fig 3C).

In contrast, the cell cycle profiles of the finite lifespan 184 HMEC cells and the immortalized MCF-10A line were not altered by ASp27 transfection. Repeat assays showed that the cyclin E1-associated kinase remained inhibited in G1 arrested ASp27-transfected 184 HMEC, as it did in TGF- β treated lipid and mismatch controls (Fig 3C). Thus, p27 is required to maintain G1 arrest by TGF- β in these tumor-derived lines but not in finite lifespan nor immortal, non-malignant mammary epithelial cells.

The failure of antisense-p27 transfected 184 HMEC and MCF-10A cells to re-enter the cell cycle was not due to toxicity, since replacement of the TGF- β containing media with complete media (no TGF- β) led to cell cycle re-entry (data not shown). Since early passage and late passage HMEC cells differ in their responsiveness to TGF- β ¹⁶⁵, we repeated the ASp27 transfection experiments with mid-passage (passage 15) and late passage (passage 20) 184

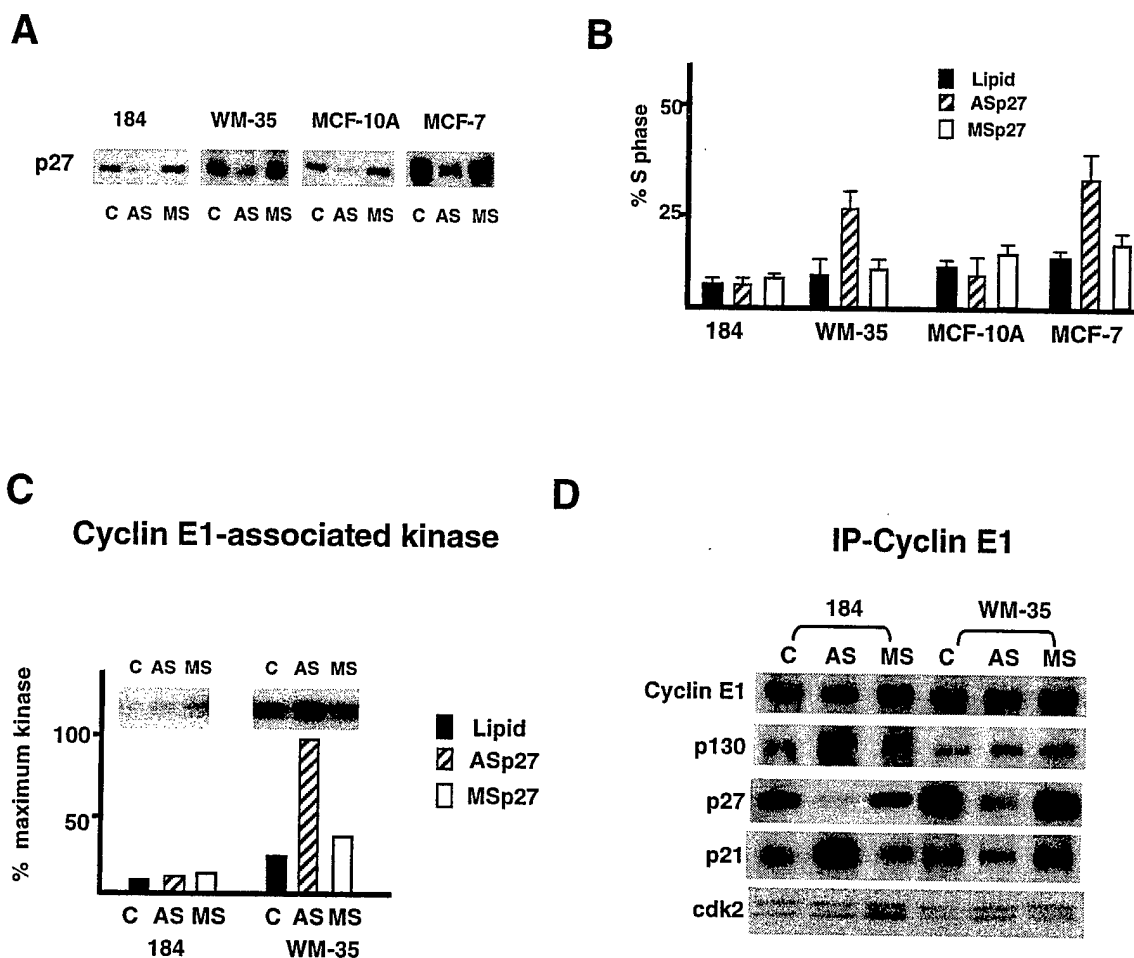


Figure 3. Maintenance of TGF- β mediated G1 arrest in the HMEC, but not malignant tumor-derived lines following p27 downregulation. The indicated cells were treated with TGF- β for 36 hours followed by a 6 hr transfection with lipid only (L), antisense p27 oligonucleotides (AS) and mismatch (MS) oligonucleotides. *A*, The levels of p27 and *B*, cell cycle profile were assayed 24 hours post-transfection. *C*, The cyclin E1-associated kinase activities and *D*, cyclin E1-bound p21, p27, p130 and Cdk2 were assayed in 184 and WM35 cells 24 hours post-transfection.

HMEC. Regardless of passage, HMEC cells maintained G1 arrest in the presence of TGF- β . The failure of the malignant tumor-derived lines to maintain G1 arrest following ASp27 was unlikely to have been due to differences in the intrinsic TGF- β sensitivity of the cell types. The finite lifespan 184 HMEC and malignant WM35 lines had similar TGF- β sensitivity, as did the non-malignant MCF-10A and malignant MCF-7 cells lines, yet only 184 and MCF-10A cell types maintained arrested by TGF- β following p27 downregulation.

What mechanisms are responsible for normal breast cells maintaining G1 arrest following p27 loss?

To investigate mechanisms contributing to maintenance of TGF- β arrest in 184 HMEC and immortal MCF-10A, despite the antisense-mediated decrease in p27 expression, we assayed the levels of p21, p27, and p130 bound to cyclin E1 in ASp27-transfected, TGF- β -treated cells. In all of the four cell types, ASp27 treatment significantly reduced the levels of p27 in cyclin E1 complexes (shown for 184 HMEC and WM35 in Fig 3D). In the 184 HMEC, the reduction in cyclin E1-bound p27 was associated with an approximately three-to-five-fold increase in cyclin E1-bound p21 and a five-to-ten fold increase in the level of p130 in the cyclin E1 complexes (Fig 3D). TGF- β treated MCF-10A also showed an increase in cyclin E1-bound p130 and p21 following antisense-mediated loss of p27 and remained arrested. In the cancer derived MCF-7 and WM35 lines, however, there was no increase in p130 association with cyclin E1 complexes, cyclin E1-bound p21 decreased in ASp27 treated cells compared to the lipid (C) and mismatch (MS) cells and ASp27 led to cell cycle re-entry.

FURTHER DISCUSSION BY AWARDEE

Loss of sensitivity to the growth inhibitory effect of TGF- β is common in human tumor-derived cell lines and is thought to contribute to malignant tumor progression¹⁶⁶. Although

increased p27 proteolysis and TGF- β resistance have both been shown to occur early in tumorigenesis, previous work has not provided a causal link between p27 deregulation and loss of G1 arrest by TGF- β during oncogenic progression. With the exception of the increased size of p27 null mice compared to the wild type mice, the relative absence of alterations in development, differentiation and cell cycle control in p27 null mice suggests that compensation by other cell cycle regulators may occur in the absence of p27^{37-39,172}. Indeed, mouse embryonic fibroblasts (MEFs) obtained from p27 null mice retain sensitivity to many growth inhibitory stimuli, including TGF- β ³⁹. The present study demonstrates that p27 is an essential mediator of G1 arrest by TGF- β in two malignant lines, the MCF-7 breast cancer cell line and the WM35 melanoma line. Antisense mediated inhibition of p27 expression led to cyclin E1-Cdk2 reactivation and cell cycle re-entry. However, p27 was not essential for G1 arrest by TGF- β in two non-tumor derived cell types, the finite-lifespan 184 HMEC and the immortalized MCF-10A line. In these cells, a compensatory increase in binding of p21^{WAF-1/CIP-1} and p130^{Rb2} to cyclin E1-Cdk2 complexes accompanied maintenance of the G1 arrest.

A number of studies using oncogene transformed and or cancer-derived cell lines support the notion that p27 loss or deregulation is associated with impaired TGF- β arrest response. Overexpression of the Bcr-Abl kinase in human M07 cells and murine Ba/F3 cells led to the proteosomal degradation of p27 and this was associated with TGF- β resistance³⁰². Oncogenic ras activation led to cytoplasmic mislocalization of p27 and to TGF- β resistance in epithelial cell lines³⁰³. In the WM35 and 184HMEC used in this study, we have shown that overexpression of activated PKB impairs TGF- β responsiveness at least in part through PKB-mediated phosphorylation of p27 leading to its cytoplasmic mislocalization³⁰⁴. Furthermore, overexpression of the viral oncoprotein E1A in mink lung epithelial cells³⁰⁵ caused TGF- β resistance. E1A bound to and blocked the accumulation of p27 in cyclin E1-Cdk2 complexes in

response to TGF- β . In the present study, we observed differences in p27 regulation in the cancer-derived lines compared to the finite lifespan 184 HMEC. The cancer-derived lines, especially MCF-7, had a paradoxically high amount of p27 present in cyclin E1-Cdk2 complexes in asynchronously proliferating cells. In addition, cyclin E1-Cdk2 complexes from both cancer-derived lines had higher kinase activities than asynchronous 184 HMEC, despite more cyclin E1-bound p27 and p21. These data suggest that the Cdk inhibitory activity of the KIPs may be impaired in MCF-7 and WM35 cells. In the context of functional KIP deregulation, even a modest loss of p27 via antisense might have a critical effect since compensatory action by p21 may be impaired.

The p130^{Rb2} protein may play an important compensatory role to maintain checkpoints following p27 loss in several cell types, including epithelial cells as we report here, and also in fibroblasts. In p27^{-/-} MEFs, the accumulation of p130 in cyclin E-Cdk2 complexes compensated for the absence of p27 and contributed to Cdk2 inhibition and G1 arrest following either pharmacologic PI3K inhibition or serum starvation^{295,306}. Other studies support a role for p130 in the proliferative arrest by TGF- β . Herzinger et al showed an accumulation of p130 in E2F complexes and repression of E2F regulated genes during TGF- β arrest of human keratinocytes³⁰⁷. We detected p130 in cyclin E1-Cdk2 complexes from both asynchronously proliferating and TGF- β treated cells. In all cells assayed, TGF- β treatment induced a modest, yet similar, increase in the levels of cyclin E1-bound p130. Surprisingly, despite 15 to 20 fold higher p130 protein levels in the two malignant tumor-derived lines than in the non-malignant cells, the levels of p130 bound to cyclin E1-Cdk2 were five to ten-fold less in these cancer-derived lines than in the HMEC. Thus, mechanisms that regulate p130 binding to cyclin E1-Cdk2 complexes may differ between the HMEC and cancer-derived lines.

p130 deregulation has been observed, and may have independent prognostic value, in several types of human cancers³⁰⁸⁻³¹⁰. Altered p130 regulation has been reported in the context of altered p27 regulation and may contribute to loss of responses to antiproliferative stimuli. For example, the viral E1A protein can bind and inactivate both p27 and p130, and E1A overexpression leads to TGF- β resistance³⁰⁵. In addition, p27^{-/-} lymphocytes, which express lower p130 levels than p27^{-/-} MEFs, fail to commit to G1 arrest following serum starvation²⁹⁵. Thus, deregulation of both p130 and p27 may potentially lead to a loss of normal proliferative control during tumor progression. Future studies may elucidate whether p130 deregulation further stratifies for poor patient outcome among patients whose tumors show reduced p27.

Our data support a notion that deregulation of multiple G1 cell cycle regulators may be required before cells lose responsiveness to antiproliferative effects of TGF- β . Deregulation of other G1 regulators may ultimately be required before p27 is rate limiting for G1 arrest by TGF- β . p15 has been shown to cooperate with p27 in G1 arrest by TGF- β ²⁹². p15 is not required for G1 arrest by TGF- β since both MCF-10A¹⁷⁹ and WM35¹⁸⁴ retain TGF- β responsiveness despite a lack of p15 expression. Moreover, p15 loss *per se* does not make cells dependent on p27 for TGF- β mediated G1 arrest. While both MCF-10A and WM35 are p15 deficient, ASp27 abrogated TGF- β arrest in only the WM35 and not the MCF-10A. These data suggest that WM35 has undergone a disruption of the pathways that become activated by TGF- β in p15 deficient MCF-10A line as a compensatory response to p27 downregulation. One of these may be the compensatory increase in p130 binding to Cdk2. The disruption of both p130 and p15 regulation in cancers may alter the role of p27 from a redundant to essential mediator of G1 arrest by TGF- β .

c-myc plays an important role in the regulation of many G1 cell cycle proteins, including cyclin E1, p27, p21, p15 and Cdc25A, and ectopic overexpression of c-myc causes TGF- β

resistance²⁹⁹. In MCF-7, the elevated c-myc levels may contribute to the loss of induction of p15 by TGF- β . Increased c-myc may also be linked to the impaired antiproliferative role of p130^{311,312} and could contribute to the increased expression of Cdc25A⁸ in MCF-7 and WM35. Cdc25A downregulation contributes to G1 arrest by TGF- β ¹⁷⁹. The increased basal Cdc25A levels seen in MCF-7 and WM35 may contribute to the increased cyclin E1-Cdk2 activities observed in these lines. Loda and colleagues have shown increased mortality in breast cancer patients whose tumors expressed both elevated Cdc25A and low p27³¹³. In addition, there was a positive correlation between Cdk2 activity and Cdc25A expression in the breast cancers studied. Increased Cdc25A expression and activity would oppose the cyclin E1-Cdk2 inhibitory function of p27. In cancers with Cdc25A overexpression, maintenance of p27 expression and function may become critical for continued responsiveness to antiproliferative stimuli such as TGF- β .

In summary, our data support the notion that the reduction in p27 levels may be an important contributing factor in the loss of normal responsiveness to growth inhibitory stimuli during cancer progression. Importantly, the reduction in p27 levels alone may be insufficient to disrupt cell cycle arrest responses due to compensation from other cell cycle regulators. Our antisense experiments suggest that normal mammary epithelial cells maintain their antiproliferative responses at least in part through activation of the Cdk inhibitory function of p21 and p130 when p27 levels are reduced. Loss of these and other normal checkpoint controls during malignant progression may make p27 essential for G1 arrest by TGF- β .

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated estradiol promotes cell cycle re-entry associated with p21 and p27 downregulation and cyclin D/cdk4 and cyclin E-cdk2 activation
- Demonstrated p27 and p21 accumulate in cyclin E-cdk2 complexes following treatment of ER positive MCF-7 cells with the pure antiestrogen ICI 182780 or the partial antiestrogen 4-hydroxytamoxifen
- Demonstrated that antiestrogen treated leads to near complete inhibition of cyclin E-associated kinase activity in MCF-7.
- Demonstrated that p21 and p27 are both essential for the cytostatic effects of antiestrogens in MCF-7
- Developed assay for p27 immunohistochemical studies and showed strong nuclear localization of p27 in G1 arrested cells.
- Established the deregulated p27 function in the LY-2 line including increased cyclin E binding and failure to increase following antiestrogen treatment.
- Demonstrated that p27 deregulation was associated with MAPK activation in several antiestrogen resistant lines (LY-2, LCC2 and HER-2/MCF-7)
- Demonstrated that MAPK activation directly causes antiestrogen resistance in MCF-7 lines overexpressing constitutively active MEK
- Demonstrated that p27 levels are reduced in MEK clones, but that cyclin E-cdk2 binding is maintained
- Demonstrated that MEK clones like LY-2 also fail to upregulate p27 binding in cyclin E-cdk2 following antiestrogen treatment.

KEY RESEARCH ACCOMPLISHMENTS (CON'T):

- Demonstrated that p27 has altered phosphorylation in LY-2 antiestrogen resistant lines and also in MEK overexpressing MCF-7.
- Demonstrated that MEK inhibition restores antiestrogen sensitivity in antiestrogen resistant LY-2 and also in Her-2/MCF-7
- Demonstrated that restoration of antiestrogen responsiveness in (12) above is associated with accumulation of p27 in cyclin E-cdk2 complexes
- Demonstrated via a kinase assay that p27 from LY-2 and MCF-7/MEK is a poorer inhibitory of cyclin E-cdk2 *in vitro*.
- Demonstrated that the combination of MEK inhibition and antiestrogen treatment restores the phosphorylation pattern of p27.
- Began developing immunohistochemical assays for active MAPK staining in paraffin embedded formalin fixed breast cancer tissues.
- Demonstrated that p27 is a key effector of G1 arrest by TGF- β in MCF-7 and WM-35 cancer lines but not in non-malignant mammary epithelial cells (HMEC).
- Demonstrated that maintenance of G1 arrest in HMEC cells is associated with accumulation of p21 and p130 in cyclin E-cdk2 and cyclin E-cdk2 inhibition.

REPORTABLE OUTCOMES

PUBLICATIONS

- 1 **Donovan JC**, Milic A, Slingerland JM. Constitutive MEK/MAPK activation leads to p27Kip1 deregulation and antiestrogen resistance in human breast cancer cells. *J Biol Chem.* Nov 2001.
- 2 **Donovan J**, Slingerland J. Transforming growth factor-beta and breast cancer: Cell cycle arrest by transforming growth factor-beta and its disruption in cancer. *Breast Cancer Res.* 2000;2(2):116-24.
- 4 Sandhu C, **Donovan J**, Bhattacharya N, Stampfer M, Worland P, Slingerland J. Reduction of Cdc25A contributes to cyclin E1-Cdk2 inhibition at senescence in human mammary epithelial cells. *Oncogene.* 2000 Nov 9;19(47):5314-23.
- 5 Cariou S, **Donovan JC***, Flanagan WM, Milic A, Bhattacharya N, Slingerland JM. Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells. *Proc Natl Acad Sci U S A.* 2000 Aug 1;97(16):9042-6.
(*co-first author)
- 6 **Donovan JC**, Rothenstein J, Milic A, and Slingerland J. p27 is an essential mediator of G1 arrest in normal but not malignant epithelial cells. (manuscript submitted August 2002)
- 7 **Donovan JC** and Slingerland, JM. The role of ras signaling to antiestrogen resistance in breast cancer. (manuscript in preparation)
- 8 **Donovan J**, Tannock I and Slingerland J. "Cell cycle regulation in normal and malignant cells." *The Basic Science of Oncology* (4th Edition). Publication expected Fall 2002.

POSTERS/ABSTRACTS:

- 1 **Donovan J**, Milic A., Cariou S, Slingerland J. "The MAPK Pathway: New Molecular Targets in the Therapy of Hormone Resistant Breast Cancer." *Keystone Symposia On Cancer Intervention*, Durango, Colorado, Feb 28-Mar 5, 2001.
- 2 Cariou S, **Donovan J**, Flanagan M, Milic A, Bhattacharya N, Slingerland J. "The cell cycle inhibitors, p21 and p27 are essential for the therapeutic effects of antiestrogens in human breast cancer cells." *Era of Hope DOD Breast Cancer Research Program Meeting*, Atlanta, GA, June 8-11, 2000

- 3 **Donovan J**, Cariou S, Milic A, Bhattacharya N, Flanagan M, Slingerland J. "p27^{Kip1}: a key effector of Estradiol:ER signaling." Signaling 2000, Keystone Symposia, Keystone, Colorado, January 22-28, 2000.
- 4 **Donovan J**, Cariou S, Bhattacharya N, Slingerland J. "Regulation of the cell cycle inhibitor p27 by estrogens and anti-estrogens." Salk Institute Cell Cycle Meeting, San Diego, CA, June 18-22, 1999.
- 5 Rothenstein J, **Donovan J**, Slingerland, JM. p27 is a key effector of TGF- β arrest in malignant breast cancer cells. Annual Research Day, University of Toronto, June 2001

PRESENTATIONS

The MAPK Pathway: New Molecular Targets for the therapy of hormone resistant breast cancer. Conference on Hormones and Cancer, Australia, 2001

Regulation of p27 by estrogens and antiestrogens. Sunnybrook & Women's College Health Sciences Centre, Toronto, ON, October, 2001

MAPK activation contributes to tamoxifen resistance. Department of Medical Biophysics speaker. University of Toronto, April 2002.

MAPK and p27 deregulation in ER positive breast cancer cells. June 2001. University of Toronto. Sunnybrook & Women's College Health Sciences Centre.

DEGREES TO BE OBTAINED:

PhD to be conferred November 2002, Toronto Ontario

CELL LINES DEVELOPED:

MCF-7-MEK1

CHAPTER VI
CONCLUSIONS

This final Section offers a summary of the major findings of the work conducted by the awardee.

Antiestrogens cause G1 arrest in ER positive breast cancer cells

About two-thirds of newly diagnosed breast cancers express the estrogen receptor (ER), and women with ER positive breast cancers are candidates for antiestrogen therapy²¹⁵. Presently, the most widely prescribed antiestrogen is Tamoxifen (TAM). TAM has shown efficacy in reducing the incidence of contralateral breast cancer and improving survival in women with metastatic disease. More recently, tamoxifen has shown efficacy in preventing breast cancer in women at high risk for the disease³¹⁴. Unfortunately, only about 60 % of women who begin antiestrogen therapy for metastatic disease initially respond; in the remaining women the disease progresses. Invariably, the vast majority of women who initially respond to antiestrogen therapy develop resistance. In the majority of cases, this occurs without loss of the estrogen receptor²¹⁵. Understanding the mechanisms that lead to antiestrogen resistance may allow for improved therapeutic strategies to treat breast cancer.

We used the MCF-7 breast cancer cell line to study the effect of estrogens and antiestrogens on the cell cycle (Section II)³⁶. MCF-7 cells are exquisitely sensitive to estradiol and undergo cell cycle arrest in G0/G1 in estradiol-depleted charcoal stripped serum. Re-addition of estradiol to quiescent cells leads to synchronous cell cycle re-entry. Progression from G0 to the G1/S border was observed 12 hours following the addition of estradiol. Maximal S phase entry occurred by 24 hours. Estradiol mediated cell cycle entry led to cyclin D1-Cdk 4 and cyclin D1-Cdk6 activation that was maximal at 6 hours followed by activation of cyclin E1-Cdk2. Cell cycle entry was accompanied by a reduction in the total protein levels of p21 and p27 and a reduction in their association with cyclin E1-Cdk2 complexes. These data concur with other published studies^{218,236-238}.

The estradiol mediated reduction in p21 and p27 levels was essential for cell cycle entry. We showed that antisense oligonucleotides targeting p21 (ASp21) or p27 (ASp27) caused a three to five fold downregulation of their respective protein levels and also a reduction in p21 and p27 association with cyclin E1-Cdk2 complexes. Antisense p21 or antisense p27 transfection mimicked the effects of estradiol by promoting cell cycle entry. Thus, we concluded that a key effect of ER signaling is to relieve KIP-mediated inhibition of Cdk2.

We showed that antiestrogens caused G1 arrest via a mechanism that involved the accumulation of p21 and p27 in cyclin E1-Cdk2 complexes and inhibition of this kinase. Inhibition of either p21 or p27 expression in antiestrogen treated cells in antiestrogen arrested cells led to cell cycle re-entry. This was observed in cells arrested by either the antiestrogen ICI 182780, 4-hydroxy-tamoxifen or by estradiol depletion. Cell cycle entry was associated with loss of KIP binding to cyclin E-Cdk2 complexes and activation of these complexes. These data demonstrated that the cell cycle arrest by antiestrogens required p21 and p27 and also that these Cdk inhibitors are not merely upregulated as a consequence of cell cycle arrest. Breast cancers lacking p27 or p21 are likely to be antiestrogen resistant.

In the Section III, I investigated how p27 deregulation may contribute to antiestrogen resistance in human breast cancer cell lines. Although inhibition of KIP expression via antisense strategies led to resistance to G1 arrest by antiestrogens, KIP protein levels was not reduced in several cell lines selected for antiestrogen resistance ¹¹⁹. I showed that p27 deregulation contributed to the antiestrogen resistant phenotype. In the antiestrogen resistant MCF-7 derivative LY-2, I observed a modest increase in p27 protein levels in asynchronously proliferating cells. p27 from LY-2 associated with cyclin E1-Cdk2 to a greater degree than in the parental MCF-7 line in both asynchronously proliferating and antiestrogen treated cells.

Paradoxically, despite an increased binding of p27 to cyclin E-Cdk2 in LY-2 cells, cyclin E-cdk2 activity was more active. In addition, immunoprecipitated p27 protein in the LY-2 line showed associated kinase activity. In contrast, there was no detectable p27-associated kinase activity in MCF-7. These data suggest that in the LY-2 line, p27 may bind some of the cyclin E-Cdk2 but fail to inhibit its kinase activity. Moreover, in contrast the observations in the MCF-7 line, antiestrogen treatment of LY-2 did not increase p27 accumulation in cyclin E-Cdk2 complexes, and did not lead to inhibition of the kinase activity of cyclin E1-Cdk2 complexes. p27 from the antiestrogen resistant LY-2 line had altered phosphorylation, and showed reduced inhibitory activity *in vitro* against recombinant active cyclin E1-Cdk2.

I found an association between p42/p44 MAPK (Erk2/Erk1) pathway and the antiestrogen resistant phenotype in several MCF-7 derivatives, including LY-2, LCC2 and MCF-7/HER-2-18. Overexpression of MEK^{EE} in MCF-7 led to antiestrogen resistance and altered p27 function. Although total p27 protein levels were reduced in MCF-7 cells overexpressing MEK^{EE}, there was no reduction in cyclin E-Cdk2-bound p27 in asynchronously proliferating cells. Surprisingly, despite similar p27 levels in cyclin E-Cdk2 complexes, the kinase activity of these complexes in MCF-7/MEK^{EE} lines was modestly increased. As in LY-2, antiestrogens failed to cause p27 accumulation in cyclin E-Cdk2 and Cdk2 remained active in the MEK^{EE} overexpressing cells. Moreover, p27 protein from MCF-7/MEK^{EE} transfectants had altered phosphorylation and reduced inhibitory function against recombinant cyclin E1-Cdk2 *in vitro* compared to p27 protein from the parental or vector-alone MCF-7 transfectants. Thus, MEK activation contributes to antiestrogen resistance at least in part by altering the phosphorylation status and the inhibitory function of p27.

Partial inhibition of MAPK activity by addition of the MAPKK inhibitor U0126 restored antiestrogen sensitivity in LY-2 and also in antiestrogen resistant MCF-7/HER-2-18

transfectants. In both LY-2 and MCF-7/HER-2-18, the combination of U0126 (U) and the antiestrogen ICI 182780 (ICI) led to an increase in cyclin E-Cdk2 bound p27, inhibition of cyclin E-associated kinase activity and G1 arrest. This restoration of antiestrogen sensitivity was p27 dependent since antisense transfection into G1 arrested U + ICI-treated cells with antisense-p27 abrogated G1 arrest. Moreover, MEK inhibition altered p27 phosphorylation and increased the Cdk2 inhibitory function of p27. These data suggest that MEK may have an important role in the regulation of p27 inhibitory function via phosphorylation. These data strongly suggest a role for the continued development of MEK inhibitory drugs in the treatment of antiestrogen resistance.

p27 is an essential mediator of G1 arrest by TGF- β in cancer-derived lines but not in finite lifespan HMEC.

TGF- β has potent antiproliferative effects in many normal cell types, including epithelial cells and melanocytes (reviewed in²⁹¹). In contrast to non-transformed cells, many cancer-derived lines have reduced anti-proliferative responses to TGF- β or have lost responsiveness altogether. Numerous studies have shown that p27 deregulation contributes to the resistance of malignant cells to G1 arrest by TGF- β . In Section V, I tested the requirement for p27 in G1 arrest by TGF- β . I showed that p27 was an essential mediator of G1 arrest by TGF- β in two cancer derived lines, MCF-7 and WM35, but not in two non-tumor-derived human mammary epithelial cells (HMEC). TGF- β treatment of all cell lines led to an accumulation of p27 in cyclin E1-Cdk2 complexes and a reduction in cyclin E1-associated kinase activity. Transfection of antisense p27 (ASp27) oligonucleotides into TGF- β treated WM35 and MCF-7 abrogated G1 arrest by TGF- β and led to cell cycle reentry. In contrast, the non-tumor derived HMEC maintained G1 arrest by TGF- β following inhibition of p27 expression by antisense.

Maintenance of G1 arrest in these non-tumor derived cells was associated with accumulation of p21^{WAF-1/Cip1} and p130^{Rb2} in cyclin E-Cdk2 complexes and maintenance of cyclin E-Cdk2 kinase inhibition. These compensatory changes were not observed in the cancer-derived MCF-7 and WM35 lines.

Previous studies had shown that certain cell types lacking p15 retain sensitivity to the anti-proliferative effects of TGF- β . The studies summarized in Section V showed that p15 loss per se does not make cells dependent on p27 to maintain G1 arrest by TGF- β . Both WM-35 and MCF-10A lack p15 expression, yet the non-transformed MCF-10A line maintains G1 arrest by TGF- β following ASp27 transfection. Thus in non-transformed cells, both p27 and p15 may be dispensable to G1 arrest by TGF- β , likely as a result of compensation by other cell cycle regulators.

These studies also highlighted an important compensatory role for p130 in the cell cycle arrest pathway by TGF- β following in cells in which p27 expression is impaired. Two previous studies using p27 ^{-/-} mouse embryonic fibroblasts subjected to serum starvation or PI3K inhibition also showed that p130 accumulation in cyclin E-Cdk2 can compensate for the p27 null status to permit G1 arrest ^{249,306}. Our studies using epithelial cell models, together with these two previous studies using fibroblast models strongly support the notion that activation of the Cdk inhibitory function of p130 may serve as a general mechanism to maintain normal cyclin E-Cdk2 regulation following loss of p27 inhibitory function. Deregulation of p130 may lead to loss of normal antiproliferative responses in breast cancer.

Reference List

- (1) Sherr, C.J. - *Cancer Res* 2000.Jul.15.;60.(14.):3689.-95.
- (2) Morgan, D.O. *Nature* 1995, 374, 131-134.
- (3) Morgan, D.O. - *Annu.Rev.Cell Dev Biol* 1997.;13.:261.-91.
- (4) Solomon, M.J.; Kaldis, P. *Results.Probl.Cell Differ.* 1998, 22, 79-109.
- (5) Nagahara, H.; Ezhevsky, S.A.; Vocero-Akbani, A.M.; Kaldis, P.; Solomon, M.J.; Dowdy, S.F. *Proc Natl Acad Sci Usa* 1999, 96, 14961-14966.
- (6) Hoffmann, I.; Draetta, G.; Karsenti, E. *EMBO J.* 1994, 13, 4302-4310.
- (7) Jinno, S.; Suto, K.; Nagata, A.; Igarashi, M.; Kanaoka, Y.; Nojima, H.; Okayama, H. *EMBO J.* 1994, 13, 1549-1556.
- (8) Galaktionov, K.; Chen, X.; Beach, D. *Nature* 1996, 382, 511-517.
- (9) Blomberg, I.; Hoffmann, I. *Mol Cell Biol* 1999, 19, 6183-6194.
- (10) Draetta, G.; Eckstein, J. - *Biochim.Biophys Acta* 1997.Apr.18.;1332.(2.):M53.-63.
- (11) Sherr, C.J. *Cell* 1994, 79, 551-555.
- (12) Hunter, T.; Pines, J. *Cell* 1991, 66, 1071-1074.
- (13) Hunter, T.; Pines, J. *Cell* 1994, 79, 573-582.
- (14) Sherr, C.J.; Roberts, J.M. *Genes Dev.* 1999, 13, 1501-1512.
- (15) Baldin, V.; Lukas, J.; Marcote, M.J.; Pagano, M.; Draetta, G. *Genes & Dev.* 1993, 7, 812-821.
- (16) Ohtsubo, M.; Theodoras, A.M.; Schumacher, J.; Roberts, J.M.; Pagano, M. *Molecular and Cellular Biology* 1995, 2612-2624.
- (17) Gudas, J.; Payton, M.; Thukral, S.F.; Chen, E.; Bass, M.; Robinson, M.; Coats, S. - *Mol Cell Biol* 1999.Jan.;19.(1.):612.-22.
- (18) Resnitzky, D.; Gossen, M.; Bujard, H.; Reed, S. *Mol.Cell.Biol.* 1994, 14, 1669-1679.
- (19) Quelle, D.E.; Ashmun, R.A.; Shurtleff, S.A.; Kato, J.; Bar-Sagi, D.; Roussel, M.F.; Sherr, C.J. *Genes & Development* 1993, 7, 1559-1571.
- (20) Koepp, D.; Schaefer, L. Ye, U.; Keyomarsi, K.; Chu, C.; Harper, J.W.; Elledge, S.J. - *Science* 2001.Oct.5.;294.(5540.):173.-7.
- (21) Girard, F.; Strausfeld, U.; Fernandez, A.; Lamb, N. *Cell* 1991, 67, 1169-79.
- (22) Resnitzky, D.; Hengst, L.; Reed, S.I. *Molecular and Cellular Biology* 1995, 4347-4352.
- (23) Takizawa, C.; Morgan, D.O. - *Curr.Opin.Cell Biol* 2000.Dec.;12.(6.):658.-65.
- (24) Fisher, B.; Costantino, J.P.; Wickerham, D.L.; Redmond, C.K.; Kavanah, M.; Cronin, W.M. *J Natl Can Inst* 1998, 90, 1371-1388.
- (25) Hengst, L.; Gopfert, U.; Lashuel, H.A.; Reed, S.I. *Genes Dev.* 1998, 12, 3882-3888.
- (26) El-Deiry, W.S.; Tokino, T.; Velculescu, V.E.; Levy, D.; Parsons, R.; Trent, J.M.; Lin, D.; Mercer, W.E.; Kinzler, K.W.; Vogelstein, B. *Cell* 1993, 75, 817-825.
- (27) Noda, A.; Ning, Y.; Venable, S.F.; Pereira-Smith, O.M.; Smith, J.R. *Exp.Cell Res.* 1994, 211, 90-98.
- (28) Xiong, Y.; Hannon, G.; Zhang, H.; Casso, D.; Kobayashi, R.; Beach, D. *Nature* 1993, 366, 701-704.
- (29) Lee, M.-H.; Reynisdottir, I.; Massague, J. *Genes Dev.* 1995, 9, 639-649.

- (30) Polyak, K.; Kato, J.Y.; Solomon, M.J.; Sherr, C.J.; Massague, J.; Roberts, J.M.; Koff, A. *Genes Dev.* **1994**, *8*, 9-22.
- (31) Slingerland, J.; Hengst, L.; Pan, C.; Alexander, D. Stampfer, M.; Reed, S.I. - *Mol Cell Biol* 1994.Jun.;14.(6.):3683.-94.
- (32) Hengst, L.; Dulic, V.; Slingerland, J.M.; Lees, E.; Reed, S.I. *Proc.Natl.Acad.Sci.U.S.A.* **1994**, *91*, 5291-5295.
- (33) Takimoto, G.S.; Graham, J.D.; Jackson, T.A.; Tung, L.; Powell, R.L.; Horwitz, L.D.; Horwitz, K.B. *J Steroid Biochem Mol Biol* **1999**, *69(1-6)*, 45-50.
- (34) Sherr, C.J.; Roberts, J.M. *Genes Dev.* **1995**, *9*, 1149-1163.
- (35) Coats, S.; Flanagan, M.; Nourse, J.; Roberts, J.M. *Science* **1996**, *272*, 877-880.
- (36) Cariou, S.; Donovan, J.C.; Flanagan, W.M.; Milic, A.; Bhattacharya, N.; Slingerland, J.M. *Proc.Natl.Acad.Sci.U.S.A.*2000.Aug.1.;97.(16.):9042.-6. **2000**, *97*, 9042-9046.
- (37) Fero, M.L.; Rivkin, M.; Tasch, M.; Porter, P.; Carow, C.E.; Polyak, K.; Firpo, E.; Tsai, L.; Broudy, V.; Perlmutter, R.M.; Kaushansky, K.; Roberts, J.M. *Cell* **1996**, *85*, 733-744.
- (38) Kiyokawa, H.; Kineman, R.D.; Manova-Todorova, K.O.; Soares, V.C.; Hoffman, E.S.; Ono, M.; Khanam, D.; Hayday, A.C.; Frohman, L.A.; Koff, A. *Cell* **1996**, *85*, 721-732.
- (39) Nakayama, K.; Ishida, N.; Shirane, M.; Inomata, A.; Inoue, T.; Shishido, N.; Horii, I.; Loh, D.Y. *Cell* **1996**, *85*, 707-720.
- (40) Sheaff, R.J.; Groudine, M.; Gordon, M.; Roberts, J.M.; Clurman, B.E. *Genes & Dev.* **1997**, *11*, 1464-1478.
- (41) Vlach, J.; Hennecke, S.; Amati, B. *EMBO J.* **1997**, *16*, 5334-5344.
- (42) Carrano, A.; Eytan, E.; Hershko, A.; Pagano, M. - *Nat Cell Biol* 1999.Aug.;1.(4.):193.-9.
- (43) Sutterluty, H.; Chatelain, E.; Marti, A.; Wirbelauer, C.; Senften, M.; Muller, U.; Krek, W. *Nat Cell Biol.* **1999**, *1*, 207-214.
- (44) Slingerland, J.F.A.U.; Pagano, M. - *J Cell Physiol.*2000.Apr.;183.(1.):10.-7.
- (45) Carrano, A.C.; Eytan, E.; Hershko, A.; Pagano, M. *Nature Cell Biol.* **1999**, *1*, 193-199.
- (46) LaBaer, J.; Garrett, M.D.; Stevenson, L.F.; Slingerland, J.M.; Sandhu, C.; Chou, H.S.; Fattaey, A.; Harlow, E. *Genes Dev.* **1997**, *11*, 847-862.
- (47) Cheng, M.; Olivier, P.; Diehl, J.A.; Fero, M.; Roussel, M.F.; Roberts, J.M.; Sherr, C.J. *EMBO J.* **1999**, *18*, 1571-1583.
- (48) Serrano, M.; Hannon, G.J.; Beach, D. *Nature* **1993**, *366*, 704-707.
- (49) Sharpless, N.; Bardeesy, N.; Lee, K.; Carrasco, D.; Castrillon, D.; Aguirre, A.; Wu, A.; Horner, J.; DePinho, R.A. - *Nature* 2001.Sep.6.;413.(6851.):86.-91.
- (50) Hannon, G.J.; Beach, D. *Nature* **1994**, *371*, 257-261.
- (51) Sandhu, C.; Garbe, J.; Daksis, J.; Pan, C.-H.; Bhattacharya, N.; Yaswen, P.; Koh, J.; Slingerland, J.; Stampfer, M.R. *Mol.Cell Biol.* **1997**, *17*, 2458-2467.
- (52) Franklin, D.; Godfrey, V.; Lee, H.; Kovalev, G.; Schoonhoven, U.; Chen-Kiang, K.; Su, L.; Xiong, Y. - *Genes Dev* 1998.Sep.15.;12.(18.):2899.-911.
- (53) Zhou, Z.Q.F.A.; Hurlin, P.J. - *Trends.Cell Biol* 2001.Nov.;11.(11.):S10.-4.
- (54) Mateyak, M.; Obaya, A.; Sedivy, J.M. - *Mol Cell Biol* 1999.Jul.;19.(7.):4672.-83.
- (55) Vlach, J.; Hennecke, S.; Alevizopoulos, K.; Conti, D.; Amati, B. *EMBO J.* **1996**, *15*, 6595-6604.
- (56) Luscher, B. - *Gene* 2001.Oct.17.;277.(1.-2.):1.-14.
- (57) O'Hagan, R.A.; Ohh, M.; David, G.; de, A, I; Alt, .; Kaelin, W.G.; DePinho, R.A. - *Genes Dev* 2000.Sep.1.;14.(17.):2185.-91.

- (58) Warner, B.J.; Blain, S.W.; Seoane, J.; Massague, J. - *Mol Cell Biol* 1999.Sep.;19.(9.):5913.-22.
- (59) Mulcahy, L.S.; Smith, M.R.; Stacey, D.W. *Nature* 1985, 313, 241-243.
- (60) Jones, S.M.; Kazlauskas, A. *Nat Cell Biol* 2001, 3, 165-172.
- (61) Gille, H.; Downward, J. - *J Biol Chem* 1999.Jul.30.;274.(31.):22033.-40.
- (62) Kerkhoff, E; Rapp, U.R. - *Oncogene* 1998.Sep.17.;17.(11.Reviews.):1457.-62.
- (63) Cheng, M.; Sexl, V.; Sherr, C.J.; Roussel, M.F. *Proc.Natl.Acad.Sci.U.S.A.* 1998, 95, 1091-1096.
- (64) Diehl, J.A.; Zindy, F.; Sherr, C.J. *Genes Dev.* 1997, 11, 957-972.
- (65) Alt, J.R.; Cleveland, J.L.; Hannink, M.; Diehl, J.A. *Genes & Dev.* 2001, 14, 3102-3114.
- (66) Muise-Helmericks, R.C.; Grimes, H.L.; Bellacosa, A.; Malstrom, S.E.; Tsichlis, P.N.; Rosen, N. *J.Biol.Chem.* 1998, 273, 29864-29872.
- (67) Leone, G.; DeGregori, J.; Sears, R.; Jakoi, L.; Nevins, J.R. *Nature* 1997, 387, 422-426.
- (68) Takuwa, N.; Takuwa, Y. *Mol.Cell Biol.* 1997, 17, 5348-5358.
- (69) Mueller, H.; Flury, N.; Eppenberger-Castori, S.; Kueng, W.; David, F.; Eppenberger, U. *Int J Cancer* 2000, 89, 384-388.
- (70) Medema, R.H.; Kops, G.J.; Bos, J.L.; Burgering, B.M. *Nature* 2000, 404, 782-787.
- (71) Mamillapalli, R.; Gavrilova, N.; Mihaylova, V.; Tsvetkov, L; Wu, H.; Zhang, H.; Sun, H. - *Curr.Biol* 2001.Feb.20.;11.(4.):263.-7.
- (72) Keyomarsi, K.; O'Leary, N.; Molnar, G; Lees, E.F; Fingert, H.; Pardee, A.B. - *Cancer Res* 1994.Jan.15.;54.(2.):380.-5.
- (73) Wang, T.; Cardiff, R.; Zukerberg, L.; Lees, E.; Arnold, A.; Schmidt, E.V. - *Nature* 1994.Jun.23.;369.(6482.):669.-71.
- (74) Barnes, D.M. - *J Pathol* 1997.Mar.;181.(3.):267.-9.
- (75) Bukholm, I.; Bukholm, G.; Nesland, J.M. - *Int J Cancer* 2001.Jul.15.;93.(2.):283.-7.
- (76) Winters, Z.; Hunt, N.; Bradburn, M.; Royds, J; Turley, H.; Harris, A.; Norbury, C.J. - *Eur.J Cancer* 2001.Dec.;37.(18.):2405.-12.
- (77) Murakami, H.; Furihata, M.; Ohtsuki, J.; Ogoshi, S. - *Virchows Arch.*1999.Feb.;434.(2.):153.-8.
- (78) Broggini, M.; Buraggi, G; Brenna, A. Riva, L; Codegoni, A.; Torri, V.; Lissoni, A.; Mangioni, C.F.A.U.; D'Incalci, M. - *Anticancer Res* 2000.Nov.-Dec.;20.(6C.):4835.-40.
- (79) Nishioka, K.; Doki, Y.; Shiozaki, H; Yamamoto, H; Tamura, S.; Yasuda, T.; Fujiwara, Y.; Yano, M.; Miyata, H.; Kishi, K.; Nakagawa, H.; Shamma, A.; Monden, M. - *Br J Cancer* 2001.Aug.3.;85.(3.):412.-21.
- (80) Cangi, M.; Cukor, B.; Soung, P.; Signoretti, S.; Moreira, G.J.; Ranashinge, M.; Cady, B.; Pagano, M.; Loda, M. - *J Clin.Invest.*2000.Sep.;106.(6.):753.-61.
- (81) Salh, B.; Marotta, A.; Matthewson, C.; Ahluwalia, M.; Flint, J.; Owen, D.; Pelech, S. *Anticancer Res* 1999, 19, 731-740.
- (82) Dong, Y.F.; Sui, L.; Sugimoto, K; Tai, Y.; Tokuda, M. - *Int J Cancer* 2001.Jul.20.;95.(4.):209.-15.
- (83) Foster, J.S.; Henley, D.C.; Bukovsky, A.; Seth, P.; Wimalasena, J. *Mol. & Cell Biol.* 2001, 21, 794-810.
- (84) An, H.; Beckmann, M.; Reifemberger, G.; Bender, ..; Niederacher, D. - *Am.J Pathol* 1999.Jan.;154.(1.):113.-8.
- (85) Tsao, H.; Benoit, E.; Sober, A.; Thiele, C.; Haluska, F.G. - *Cancer Res* 1998.Jan.1.;58.(1.):109.-13.
- (86) Mihara, M.; Shintani, S.; Nakahara, Y.; Kiyota, A.; Ueyama, Y.; Matsumura, T.; Wong, D.T. - *Jpn.J Cancer Res* 2001.Mar.;92.(3.):352.-60.
- (87) Tsihlias, J.; Kapusta, L.; Slingerland, J. *Annu.Rev.Med.* 1999, 50, 401-423.

- (88) Cariou, S.; Catzavelos, C.; Slingerland, J.M. *Breast Cancer Res Treat* **1998**, *52*, 29-41.
- (89) Slingerland, J.; Pagano, M. - *Results.Probl.Cell Differ.*1998.;22.:133.-47.
- (90) Latres, E.; Chiarle, R.; Schulman, B.; Pavletich, N.; Pellicer, A.; Inghirami, G.; Pagano, M. - *Proc.Natl Acad.Sci.U.S.A.*2001.Feb.27.;98.(5.):2515.-20.
- (91) Gstaiger, M.; Jordan, R.; Lim, M.; Catzavelos, .; Mestan, J.; Slingerland, J.; Krek, W. - *Proc.Natl Acad.Sci.U.S.A.*2001.Apr.24.;98.(9.):5043.-8.
- (92) Hershko, D.; Bornstein, G.; Ben-Izhak, O.; Carrano, A.; Pagano, M.; Krausz, M.; Hershko, A. - *Cancer* 2001.May.1.;91.(9.):1745.-51.
- (93) Singh, S.P.; Lipman, J.; Goldman, H.; Ellis, F.H.; Aizenman, L.; Cangi, M.G.; Signoretti, S.; Chiaur, D.S.; Pagano, M.; Loda, M. *Cancer Research* **1998**, *58*, 1730-1735.
- (94) Zhou, B.P.; Liao, Y.; Xia, W.; Spohn, B.; Lee, M.H.; Hung, M.C. *Nat Cell Biol* **2001** , *3*, 245-252.
- (95) Li, Y.; Dowbenko, D.; Lasky, L.A. - *J Biol Chem* 2002.Mar.29.;277.(13.):11352.-61.
- (96) Rossig, L.; Badorff, C.; Holzmann, Y.; Zeiher, A.; Dimmeler, S. - *J Biol Chem* 2002.Mar.22.;277.(12.):9684.-9.
- (97) Ruas, M.; Peters, G. *Biochim.Biophys.Acta* **1998**, *1378*, F115-177.
- (98) Wong, I; Ng, M.; Huang, D.; Lee, J.C. - *Blood* 2000.Mar.15.;95.(6.):1942.-9.
- (99) Latres, E.; Malumbres, M.; Sotillo, R.; Martin, J.; Ortega, S.; Martin-Caballero, J.; Flores, J.; Cordon-Cardo, C.; Barbacid, M. - *EMBO J* 2000.Jul.3.;19.(13.):3496.-506.
- (100) Zindy, F; den Besten, W.; Chen, B.; Rehg, J; Latres, E.; Barbacid, M.; Pollard, J.; Sherr, C.J.; Cohen, P; Roussel, M.F. - *Mol Cell Biol* 2001.May.;21.(9.):3244.-55.
- (101) Prall, O.; Rogan, E.M.; Sutherland, R.L. - *J Steroid Biochem Mol Biol* 1998.Apr.;65.(1.-6.):169.-74.
- (102) Klein-Hitpass, L.; Ryffel, G.; Heitlinger, E.; Cato, A.C. - *Nucleic.Acids.Res* 1988.Jan.25.;16.(2.):647.-63.
- (103) Kumar, V.; Chambon, P. - *Cell* 1988.Oct.7.;55.(1.):145.-56.
- (104) Lukas, J.; Bartkova, J; Bartek, J. - *Mol Cell Biol* 1996.Dec.;16.(12.):6917.-25.
- (105) Watson, P.H.; Pon, R.T.; Shiu, R.P. *Cancer Research* **1991**, *51*, 3996-4000.
- (106) Burgin, A.; Bouchard, C.; Eilers, M. - *Results.Probl.Cell Differ.*1998.;22.:181.-97.
- (107) Robertson, J.F. - *Br J Cancer* 2001.Nov.;85.Suppl.2.:11.-4.
- (108) Anonymous - *Lancet* 1998.May.16.;351.(9114.):1451.-67.
- (109) Makris, A.; Powles, T.; Allred, D.; Ashley, S; Ormerod, M.; Titley, J.; Dowsett, M. - *Breast Cancer Res Treat* 1998.Mar.;48.(1.):11.-20.
- (110) Chang, J.; Powles, T.; Allred, D.; Ashley, ; Makris, A.; Gregory, R; Osborne, C.K.; Dowsett, M. - *Clin. Cancer Res* 2000.Feb.;6.(2.):616.-21.
- (111) Johnston, S.; Saccani-Jotti, G.; Smith, I.; Salter, J.; Newby, J.; Coppen, M.; Ebbs, S.; Dowsett, M. - *Cancer Res* 1995.Aug.1.;55.(15.):3331.-8.
- (112) Herman, M.; Katzenellenbogen, B.S. - *J Steroid Biochem Mol Biol* 1996.Oct.;59.(2.):121.-34.
- (113) Musgrove, E.A.; Hamilton, J.A.; Lee, C.S.L.; Sweeney, K.J.E.; Watts, C.K.W.; Sutherland, R.L. *Mol.Cell.Biol.* **1993**, *13*, 3577-3587.
- (114) Wilcken, N.R.; Prall, W.W.J.; Musgrove, E.A.; Sutherland, R.L. *Clinical Cancer Research* **1997**, *3*, 849-854.
- (115) Liu, E.; Santos, G.; Lee, W.; Osborne, C.K.; Benz, C.C. - *Oncogene* 1989.Aug.;4.(8.):979.-84.

- (116) Levin, E.R. - *Trends.Endocrinol.Metab.*1999.Nov.;10.(9.):374.-377.
- (117) Migliaccio, ; DiDomenico, M.; Castona, C.; DeFalco, A.; Bontempo, P.; Nola, E.; Auricchio, F. *EMBO J.* 1996, 15, 1292-300.
- (118) Lobenhofer, E.; Huper, G.; Iglehart, J.; Marks, J.R. - *Cell Growth Differ.*2000.Feb.;11.(2.):99.-110.
- (119) Donovan, J.C.; Milic, A.; Slingerland, J.M. *J.Biol.Chem.* 2001, 276, 40888-40895.
- (120) Gee, J.M.; Robertson, J.; Ellis, I; Nicholson, R.I. - *Int J Cancer* 2001.Jul.20.;95.(4.):247.-54.
- (121) Perez-Tenorio, G.; Stal, O. - *Br J Cancer* 2002.Feb.12.;86.(4.):540.-5.
- (122) Joel, P.B.; Traish, A.M.; Lannigan, D.A. *J Biol Chem* 1998, 273, 13317-13323.
- (123) Kato, S.; Endoh, H.; Masuhiro, Y.; Kitamoto, T.; Uchiyama, S.; Sasaki, H.; Masushige, S.; Gotoh, Y.; Nishida, E.; Kawashima, H.; Metzger, D.; Chambon *Science* 1995, 270, 1491-1494.
- (124) Bunone, G.; Briand, P.A.; Miksicek, R.J.; Picard, D. *EMBO J* 1996, 15, 2174-2183.
- (125) Castoria, G.; Migliaccio, A.; Bilancio, A.; Di Domenico, M.; de Falco, A.; Lombardi, M.; Fiorentino, R.; Varricchio, L.; Barone, M.; Auricchio, F. - *EMBO J* 2001.Nov.1.;20.(21.):6050.-9.
- (126) Watanabe, G.; Howe, A.; Lee, R.; Albanese, C.; Shu, L.; Karnezis, A.; Zon, L.; Kyriakis, J.; Rundell, K. Pestell, R.G. - *Proc.Natl Acad.Sci.U.S.A.*1996.Nov.12.;93.(23.):12861.-6.
- (127) Meyerson, M.; Harlow, E. - *Mol Cell Biol* 1994.Mar.;14.(3.):2077.-86.
- (128) Porter, P.L.; Malone, K.E.; Heagerty, P.J.; Alexander, G.M.; Gatti, L.A.; Firpo, E.J.; Daling, J.R.; Roberts, J.M. *Nature Med.* 1997, 3, 222-225.
- (129) Rivard, N.; Boucher, M.; Asselin, C.; L'Allemain, G. - *Am.J Physiol.*1999.Oct.;277.(4.Pt.1.):C652.-64.
- (130) Kawada, M.; Yamagoe, S.; Murakami, Y.; Suzuki, K.; Mizuno, S.; Uehara, Y. *Oncogene* 1997, 15, 629-637.
- (131) Sears, R.; Leone, G.; DeGregori, J.; Nevins, J.R. - *Mol Cell* 1999.Feb.;3.(2.):169.-79.
- (132) Bouchard, C.; Thieke, K.; Maier, A.; Saffrich, R.; Hanley-Hyde, J.; Ansorge, W.; Reed, S.; Sicinski, P.; Bartek, J.; Eilers, M. *EMBO J.* 1999, 18, 5321-5333.
- (133) Perez-Roger, I.; Kim, S.H.; Griffiths, B.; Sweing, A.; Land, H. *EMBO J* 1999, 18, 5310-5320.
- (134) Clark, G.J.F.A.; Der, C.J. - *Breast Cancer Res Treat* 1995.Jul.;35.(1.):133.-44.
- (135) Dickson, R.; Kasid, A.; Huff, K.; Bates, S.; Knabbe, C.; Bronzert, D.; Gelmann, E.; Lippman, M.E. - *Proc.Natl Acad.Sci.U.S.A.*1987.Feb.;84.(3.):837.-41.
- (136) Sukumar, S.; Carney, W.P.; Barbacid, M. - *Science* 1988.Apr.22.;240.(4851.):524.-6.
- (137) Graus-Porta, D.; Beerli, R.; Daly, J.M.; Hynes, N.E. - *EMBO J* 1997.Apr.1.;16.(7.):1647.-55.
- (138) Pawson, T.F.A.U.; Scott, J.D. - *Science* 1997.Dec.19.;278.(5346.):2075.-80.
- (139) Sainsbury, J.R.; Farndon, J.R.; Needham, G.K.; Malcom, A.J.; A.L., H. *Lancet* 1987, 1, 1398-402.
- (140) Bolla, M.; Chedin, M.F.; Souvignet, C.; Marron, J.; Arnould, C.; Chambaz, E. - *Breast Cancer Res Treat* 1990.Sep.;16.(2.):97.-102.
- (141) Toi, M.; Osaki, A.; Yamada, H.; Toge, T. - *Eur.J Cancer* 1991.;27.(8.):977.-80.
- (142) Miller, D.; el-Ashry, D.F; Cheville, A.; Liu, Y; McLeskey, S.; Kern, F.G. - *Cell Growth Differ.*1994.Dec.;5.(12.):1263.-74.
- (143) van Agthoven, T.F.; van Agthoven, T.L.; Portengen, H.F.; Foekens, J.A.F.A.; Dorssers, L.C. - *Cancer Res* 1992.Sep.15.;52.(18.):5082.-8.

- (144) McClelland, R.A.; Barrow, D.; Madden, T.; Dutkowski, C.; Pamment, J.; Knowlden, J.; Gee, J.M.; Nicholson, R.I. - *Endocrinology* 2001.Jul.;142.(7.):2776-88.
- (145) Newby, J.; Johnston, S.; Smith, I.; Dowsett, M. - *Clin.Cancer Res* 1997.Sep.;3.(9.):1643-51.
- (146) Slamon, D.J.; Clark, G.M.; Wong, S.G.; Levin, W.J.; Ullrich, A.; McGuire, W.L. *Science* 1987, 235, 177-82.
- (147) Hynes, N.; Stern, D.F. - *Biochim.Biophys Acta* 1994.Dec.30.;1198.(2.-3.):165-84.
- (148) Muss, H.B. - *Semin.Oncol* 2001.Aug.;28.(4.):313-21.
- (149) Elledge, R.; Green, S.F.; Ciocca, D.F.; Pugh, R.F.; Allred, D.; Clark, G.; Hill, J.F.; Ravdin, P.F.A.U.; O'Sullivan, J.F.; Martino, S.F.; Osborne, C.K. - *Clin.Cancer Res* 1998.Jan.;4.(1.):7-12.
- (150) Looi, L.M.F.A.; Cheah, P.L. - *Malays.J Pathol* 1998.Jun.;20.(1.):19-23.
- (151) Pietras, R.J.; Arboleda, J.; Reese, D.M.; Wongvipat, N.; Pegram, M.D.; Ramos, L.; Gorman, C.M.; Parker, M.G.; Sliwkowski, M.X.; Slamon, D.J. *Oncogene* 1995, 10, 2435-2446.
- (152) Benz, C.C.; Scott, G.K.; Sarup, J.C.; Johnson, R.M.; Tripathy, D.; Coronado, E.; Shepard, H.M.; Osborne, C.K. *Breast Cancer Res Treat* 1992, 24, 85-95.
- (153) Lupu, R.; Lippman, M.E. - *Breast Cancer Res Treat* 1993.;27.(1.-2.):83-93.
- (154) Gille, J.; Swerlick, R.A.F.A.; Caughman, S.W. - *EMBO J* 1997.Feb.17.;16.(4.):750-9.
- (155) Riese, D.J.; Stern, D.F. - *Bioessays* 1998.Jan.;20.(1.):41-8.
- (156) Alroy, I.; Yarden, Y. - *FEBS Lett* 1997.Jun.23.;410.(1.):83-6.
- (157) Newman, L.; Xia, W.; Yang, H.; Sahin, A.; Bndy, M.; Lukmanji, F.F.A.U.; Hung, M.; Lee, M.H. - *Mol Carcinog* 2001.Mar.;30.(3.):169-75.
- (158) Chappuis, P.; Kapusta, L.; Begin, L.; Wong, N.; Brunet, J.; Narod, S.; Slingerland, J.; Foulkes, W.D. - *J Clin.Oncol* 2000.Dec.15.;18.(24.):4045-52.
- (159) Massague, J.; Cheifetz, S.; Laiho, M.; Ralph, D.A.; Weis, F.M.B.; Zentella, A. *Cancer Surv.* 1992, 12, 81-103.
- (160) Alexandrow, M.G.; Moses, H.L. *Cancer Res.* 1995, 55, 1452-1457.
- (161) Daniel, C.W.; Silberstein, G.B.; van Horn, K.; Strickland, P.; Robinson, S. *Dev.Biol.* 1989, 135, 20-30.
- (162) Silberstein, G.B.; Daniel, C.W. *Science* 1987, 237, 291-293.
- (163) Pierce, D.F.J.; Johnson, M.D.; Matzui, Y.; Robinson, S.D.; Gold, L.I.; Purchio, A.F.; Daniel, C.W.; Hogan, B.L.M.; Moses, H. *Genes & Development* 1993, 7, 2308-2317.
- (164) Pierce, D.F.; Gorska, A.E.; Chytil, A.; Meise, K.S.; Page, D.L.; Coffey, R.J.; Moses, H. *Proc.Natl.Acad.Sci.USA* 1995, 92, 4254-4258.
- (165) Hosobuchi, M.; Stampfer, M.R. *In Vitro Cell Dev.Biol.* 1989, 25, 705-713.
- (166) Fynan, T.M.; Reiss, M. *Crit.Rev.Oncog.* 1993, 4, 493-540.
- (167) Massague, J. *Annu.Rev.Biochem.* 1998, 67, 753-791.
- (168) Kerbel, R.S. *Am.J.Pathol.* 1992, 141, 519-524.
- (169) Laiho, M.; DeCaprio, J.A.; Ludlow, J.W.; Livingston, D.M.; Massague, J. *Cell* 1990, 62, 175-185.
- (170) Howe, P.H.; Draetta, G.; Leof, E.B. *Mol.Cell Biol.* 1991, 11, 1185-1194.
- (171) Slingerland, J.M.; Hengst, L.; Pan, C.-H.; Alexander, D.; Stampfer, M.R.; Reed, S.I. *Mol.Cell.Biol.* 1994, 14, 3683-3694.

- (172) Koff, A.; Ohtsuki, M.; Polyak, K.; Roberts, J.M.; Massague, J. *Science* **1993**, *260*, 536-539.
- (173) Schwarz, J.K.; Bassing, C.H.; Kovesdi, I.; Datto, M.B.; Blazing, M.; George, S.; Wang, X.-F.; Nevins, J.R. *Proc Natl Acad Sci Usa* **1995**, *92*, 483-487.
- (174) Pietenpol, J.A.; Stein, R.W.; Moran, E.; Yaciuk, P.; Schlegel, R.; Lyons, R.M.; Pittelkow, M.R.; Munger, K.; Howley, P.M.; Moses, H.L. *Cell* **1990**, *61*, 777-785.
- (175) Coffey, R.J.; Bascom, C.C.; Sipes, N.J.; Graves-Deal, R.; Weissman, B.E.; Moses, H. *Molec.Cell Biol.* **1988**, *8*, 3088-3093.
- (176) Alexandrow, M.G.; Kawabata, M.; Aakre, M.; Moses, H. *Proc Natl Acad Sci Usa* **1995**, *92*, 3239-3243.
- (177) Jansen-Durr, P.; Meichle, A.; Steiner, P.; Pagano, M.; Finke, K.; Botz, J.; Wessbecher, J.; Draetta, G.; Eilers, M. *Proc Natl Acad Sci Usa* **1993**, *90*, 3685-3690.
- (178) Shibuya, H.J.; Yoneyama, M.; Ninomiya-Tsuji, J.; Matsumoto, K.; Taniguchi, T. *Cell* **1992**, *70*, 57-67.
- (179) Iavarone, A.; Massague, J. *Nature* **1997**, *387*, 417-422.
- (180) Warner, B.J.; Blain, S.W.; Seoane, J.; Massague, J. *Mol.Cell Biol.* **1999**, *19*, 5913-5922.
- (181) Geng, Y.; Weinberg, R.A. *Proc.Natl.Acad.Sci.USA.* **1993**, *90*, 10315-10319.
- (182) Feng, X.-H.; Filvaroff, E.H.; Derynck, R. *J Cell Biol Chem* **1995**, *270*, 24237-24245.
- (183) Ko, T.C.; Sheng, H.M.; Reisman, D.; Thompson, E.A.; Beauchamp, R.D. *Oncogene* **1995**, *10*, 177-184.
- (184) Florenes, V.A.; Bhattacharya, N.; Bani, M.R.; Ben-David, Y.; Kerbel, R.S.; Slingerland, J.M. *Oncogene* **1996**, *13*, 2447-2457.
- (185) Reynisdottir, I.; Polyak, K.; Iavarone, A.; Massague, J. - *Genes Dev* **1995**, *Aug.1.;9(15.):1831.-45*.
- (186) Li, J.M.; Nichols, M.A.; Chandrasekharan, S.; Xiong, Y.; Wang, X.F. *J.Biol.Chem.* **1995**, *270*, 26750-26753.
- (187) Datto, M.B.; Li, Y.; Panus, J.F.; Howe, D.J.; Xiong, Y.; Wang, X.F. *Proc.Natl.Acad.Sci.U.S.A.* **1995**, *92*, 5545-5549.
- (188) Malliri, A.; Yeudall, W.A.; Nikolic, M.; Crouch, D.H.; Parkinson, E.K.; Ozanne, B. *Cell Growth Differ.* **1996**, *7*, 1291-1304.
- (189) Elbendary, A.; Berchuck, A.; Davis, P.; Havrilesky, L.; Bast, R.C., Jr.; Iglehart, J.D.; Marks, J.R. *Cell Growth Diff.* **1994**, *12*, 1301-1307.
- (190) Hunt, K.K.; Fleming, J.B.; Abramian, A.; Zhang, L.; Evans, D.B.; Chiao, P.J. *Cancer Research* **1998**, *58*, 5656-5661.
- (191) Datto, M.B.; Hu, P.P.; Kowalik, T.F.; Yingling, J.; Wang, X.F. *Mol Cell Biol* **1997**, *17*, 2030-2037.
- (192) Kato, J.Y.; Matsuoka, M.; Polyak, K.; Massague, J.; Sherr, C.J. *Cell* **1994**, *79*, 487-496.
- (193) Draetta, G.; Eckstein, J. *Biochim.Biophys.Acta* **1997**, *1332*, M53-63.
- (194) Cairns, P.; Mao, L.; Merlo, A.; Lee, D.; Schwab, D.; Eby, Y.; Tokino, K.; van der Riet, P.; Blaugrund, J.E.; Sidransky, D. *Science* **1994**, *265*, 415-417.
- (195) Cairns, P.; Polascik, T.J.; Eby, Y.; Tokino, K.; Califano, J.; Merlo, A.; Mao, L.; Herath, J.; Jenkins, R.; Westra, W.; Rutter, J.L.; Buckler, A.; Gabrielson, E.; Tockman, M.; Cho, K.R.; Hedrick, L.; Bova, G.S.; Isaacs, W.; Koch, W.; Schwab, D.; Sidransky, D. *Nature Genetics* **1995**, *11*, 210-212.
- (196) Kamb, A.; Shattuch-Eidens, D.; Eetes, R.; Lui, Q.; Gruis, N.A.; Ding, W.e.al. *Nat.Genetics.* **1994**, *8*, 23-26.
- (197) Batova, A.; Diccianni, M.B.; Yu, J.C.; Nobori, T.; Link, M.P.; Pullen, J.; Yu, A.L. *Cancer Research* **1997**, *57*, 832-836.
- (198) Quesnel, B.; Guillermin, G.; Verecque, R.; Wattel, E.; Preudhomme, C.; Bauters, F.; Vanrumbeke, M.; Fenaux, P. *Blood* **1998**, *91*, 2985-2990.
- (199) Lammie, G.A.; Fantl, V.; Smith, R.; Schuurin, E.; Brokes, S.; Dickson, R.; Arnold, A.; Peters, G. *Oncogene* **1991**, *6*, 439-444.

- (200) Buckley, M.F.; Sweeney, K.J.E.; Hamilton, J.A.; Sini, R.L.; Manning, D.L.; Nicholson, R.I.; deFazio, A.; Watts, C.K.W.; Musgrove, E.A.; Sutherland, R.L. *Oncogene* **1993**, *8*, 2127-2133.
- (201) Okamoto, A.; Jiang, W.; Kim, S.J.; Spillare, E.A.; Stoner, G.D.; Weinstein, I.B.; Harris, C.C. *Proc Natl Acad Sci Usa* **1994**, *91*, 11576-11580.
- (202) Nielsen, N.; Arnerlov, C.; Emdin, S.; Landberg, G. *British Journal Of Cancer* **1996**, *74*, 874-880.
- (203) Ewen, M.; Sluss, H.; Whitehouse, L.; Livingston, D.M. - *Cell* **1993.Sep.24**, *74*(6.):1009-20.
- (204) An, H.-X.; Beckmann, M.W.; Reifemberger, G.; Bender, H.G.; Niederacher, D. *Am J Path* **1999**, *154*, 113-118.
- (205) Bates, S.; Peters, G. *Seminars in Cancer Biology* **1995**, *6*, 73-82.
- (206) Horowitz, J.M.; Park, S.H.; Bogenmann, E.; Cheng, J.C.; Yandell, D.W.; Kaye, F.J.; Minna, J.D.; Dryja, T.P.; Weinberg, R.A. *Proc Natl Acad Sci Usa* **1990**, *87*, 2775-2779.
- (207) Filmus, J.; Zhao, J.; Buick, R.N. *Oncogene* **1992**, *7*, 521-526.
- (208) Kretzschmar, M.; Doody, J.; Timokhina, I.; Massague, J. *Genes Dev.* **1999**, *13*, 804-816.
- (209) Aktas, H.; Cai, H.; Cooper, G.M. *Mol.Cel.Biol.* **1997**, *17*, 3850-3857.
- (210) Diehl, J.A.; Cheng, M.; Roussel, M.F.; Sherr, C.J. *Genes Dev.* **1998**, *12*, 3499-3511.
- (211) Brennan, P.; Babbage, J.W.; Burgering, B.M.T.; Groner, B.; Reif, K.; Cantrell, D.A. *Immunity* **1997**, *7*, 679-689.
- (212) Musgrove, E.A.; Sutherland, R.L. *Cancer Biology* **1994**, *5*, 381-389.
- (213) Henderson, B.E.; Roos, R.; Bernstein, L. *Cancer Research* **1988**, *48*, 246-253.
- (214) Anonymous - *Lancet* **1998.May.16**, *351*(9114.):1451.-67. **1998**,
- (215) Jordan, V.C. *Breast Cancer Res Treat* **1995**, *36*, 267-285.
- (216) Sutherland, R.L.; Green, M.D.; Hall, R.E.; Reddel, R.R.; Taylor, I.W. *Eur.J.Cancer Clin.Oncol.* **1983**, *19*, 615-621.
- (217) Osborne, C.K.; Boldt, D.H.; Clark, G.M.; Trent, J.M. *Cancer Res.* **1983**, *43*, 3583-3585.
- (218) Watts, C.K.W.; Brady, A.; Sarcevic, B.; deFazio, A.; Sutherland, R.L. *Mol.End.* **1996**, *9*, 1804-13.
- (219) Nicholson, R.I.; Francis, A.B.; McClelland, R.A.; Manning, D.L.; Gee, J.M.W. *Endocrine-Related Cancer* **1994**, *3*, 1-13.
- (220) Encarnacion, C.A.; Ciocca, D.R.; McGuire, W.L.; Clark, G.M.; Fuqua, S.A.; Osborne, C.K. *Breast Cancer Res Treat* **1993**, *26*, 237-246.
- (221) Robertson, J.F.R. *British Journal Of Cancer* **1996**, *73*, 5-12.
- (222) Howell, A.; DeFriend, D.; Robertson, J.; Blamey, R.; Walton, P. *Lancet* **1995**, *245*, 29-30.
- (223) Howell, A.; DeFriend, D.J.; Robertson, J.F.R.; Blamey, R.W.; Anderson, L.; Anderson, E.; Sutcliffe, F.A.; Walton, P. *British Journal Of Cancer* **1996**, *74*, 300-308.
- (224) Perlmann, T.; Evans, R.M. *Cell* **1997**, *90*, 391-397.
- (225) Beato, M.; Chavez, S.; Truss, M. *Steroids* **1996**, *61*, 240-251.
- (226) Katzenellenbogen, J.A.; O'Malley, B.W.; Katzenellenbogen, B.S. *Mol.Endocrinol.* **1996**, *10*, 119-131.
- (227) Collins, P.; Webb, C. *Nature Medicine* **1999**, *5*, 1130-1131.
- (228) van der Kwast, T.H.; Schalken, J.; Ruizeveld de Winter, J.A.; van Vroonhoven, C.C.; Mulder, E.; Boersma, W.; Trapman, J. *Int.J.Cancer* **1991**, *48*, 189-193.

- (229) Ruizeveld de Winter, J.A.; Janssen, P.J.; Sleddens, H.M.; Verleun-Mooijman, M.C.; Trapman, J.; Brinkmann, A.O.; Santerse, A.B.; Schroder, F.H.; van der Kwast, T.H. *Am.J.Pathol.* **1994**, *144*, 735-746.
- (230) Reed, S.I.; Bailly, E.; Dulic, V.; Hengst, L.; Resnitzky, D.; Slingerland, J. *Journal of Cell Science* **1994**, *Supplement 18*, 69-73.
- (231) Catzavelos, C.; Bhattacharya, N.; Ung, Y.C.; Wilson, J.A.; Roncari, L.; Sandhu, C.; Shaw, P.; Yeger, H.; Morava-Protzner, I.; Kapusta, L.; Franssen, E.; Pritchard, K.I.; Slingerland, J.M. *Nature Med.* **1997**, *3*, 227-230.
- (232) Tan, P.; Cady, B.; Wanner, M.; Worland, P.; Cukor, B.; Magi-Galluzzi, C.; Lavin, P.; Draetta, G.; Pagano, M.; Loda, M. *Cancer Res.* **1997**, *57*, 1259-1263.
- (233) Wakasugi, E.; Kobayashi, T.; Tamaki, Y.; Ito, Y.; Miyashiro, I.; Konoike, Y.; Takeda, T.; Shin, E.; Takatsuka, Y.; Kikkawa, N.; Monden, T.; Monden, M. *Am.J.Clin.Pathol.* **1997**, *107*, 684-691.
- (234) Jiang, M.; Shao, Z.-M.; Wu, J.; Lu, J.-S.; Yu, L.-M.; Yuan, J.-D.; Han, Q.-X.; Shen, Z.-Z.; Fontana, J.A. *Int.J.Cancer* **1997**, *74*, 529-534.
- (235) Saez, A.; Sanchez, E.; Sanchez-Beato, M.; Cruz, M.A.; Chacon, I.; Munoz, E.; Camacho, F.I.; Martinez-Montero, J.C.; Mollejo, M.; Garcia, J.F.; Piris, M.A. *Br.J.Cancer* **1999**, *80*, 1427-1434.
- (236) Prall, O.W.J.; Sarcevic, B.; Musgrove, E.A.; Watts, C.K.W.; Sutherland, R.L. *J.Biol.Chem.* **1997**, *272*, 10882-10894.
- (237) Planas-Silva, M.D.; Weinberg, R.A. *Mol.Cell Biol.* **1997**, *17*, 4059-4069.
- (238) Foster, J.; Wimalasen, J. *Mol.End.* **1996**, *10*, 488-96.
- (239) Soule, H.D.; Vazquez, J.; Long, A.; Albert, S.; Brennan, M. *J.Natl.Cancer Inst.* **1973**, *51*, 1409-1416.
- (240) Petrocelli, T.; Poon, R.; Drucker, D.; Slingerland, J.; Rosen, C. *Oncogene* **1996**, *12*, 1387-1396.
- (241) Flanagan, W.M.; Wolf, J.J.; Olson, P.; Grant, D.; Lin, K.Y.; Wagner, R.W.; Matteucci, M.D. *Proc.Natl.Acad.Sci.U.S.A.* **1999**, *96*, 3513-3518.
- (242) St.Croix, B.; Florenes, V.; Rak, A.; Flanagan, J.W.; Bhattacharya, N.; Slingerland, J.M.; Kerbel, R.S. *Nature Medicine* **1996**, *2*, 1204-1210.
- (243) Musgrove, E.; Lilischkis, R.; Cornish, A.L.; Lee, S.L.; Setlur, V.; Seshari, R.; Sutherland, R.L. *Int.J.Cancer* **1995**, *63*, 584-591.
- (244) Watts, C.K.; Sweeney, K.J.E.; Warlters, A.; Musgrove, E.A.; Sutherland, R.L. *Breast Cancer Res Treat* **1994**, *31*, 95-105.
- (245) Prall, O.W.J.; Rogan, E.M.; Musgrove, E.A.; Watts, C.K.W.; Sutherland, R.L. *Molecular & Cellular Biology* **1998**, *18*, 4499-4508.
- (246) Sheaff, R.J.; Singer, J.D.; Swanger, J.; Smitherman, M.; Roberts, M.J.; Clurman, B.E. *Molecular Cell* **2000**, *5*, 403-410.
- (247) Montagnoli, A.; Fiore, F.; Eytan, E.; Carrano, A.C.; Draetta, G.F.; Hershko, A.; Pagano, M. *Genes & Dev.* **1999**, *13*, 1181-1189.
- (248) Rivard, N.; L'Allemain, G.; Bartek, J.; Pouyssegur, J. *J.Biol.Chem.* **1996**, *271*, 18337-18341.
- (249) Coats, S.; White, P.; Fero, M.L.; Lacy, S.; Chung, G.; Randel, E.; Firpo, E.; Roberts, J.M. *Current Biology* **1999**, *9*, 163-173.
- (250) Bates, S.; Parry, D.; Bonetta, L.; Vousden, K.; Dickson, C.; Peters, G. *Oncogene* **1994**, *9*, 1633-1640.
- (251) Parry, D.; Bates, S.; Mann, D.J.; Peters, G. *EMBO J.* **1995**, *14*, 503-511.
- (252) Matsushime, H.; Quelle, D.E.; Shurtleff, S.A.; Shibuya, M.; Sherr, C.J.; Kato, J.Y. *Mol.Cell.Biol* **1994**, *14*, 2066-2076.
- (253) Pagano, M.; Tam, S.W.; Theodoras, A.M.; Beer-Romero, P.; Del Sal, G.; Chau, V.; Yew, P.R.; Draetta, G.F.; Rolfe, M. *Science* **1995**, *269*, 682-685.
- (254) Tsvetkov, L.M.; Yeh, K.H.; Lee, S.J.; Sun, H.; Zhang, H. *Curr Biol* **1999**, *9*, 661-664.
- (255) Alessandrini, A.; Chiaur, D.S.; Erikson, R.; Pagano, M. *Leukemia* **1997**, *11*, 342-345.
- (256) Early Breast Cancer Trialists' Collaborative Group *The Lancet* **1998**, *351*, 1451-1467.

- (257) Sandhu, C.; Garbe, J.; Bhattacharya, N.; Daksis, J.; Pan, C.; Yaswen, P.; Koh, J.; Slingerland, J.M.; Stampfer, M.R. - *Mol Cell Biol* 1997.May.;17.(5.):2458-67.
- (258) Hengst, L.; Reed, S.I. *Science* 1996, 271, 1861-1864.
- (259) Millard, S.S.; Yan, J.S.; Nguyen, H.; Pagano, M.; Kiyokawa, H.; Koff, A. *J.Biol.Chem.* 1997, 272, 7093-7098.
- (260) Ponce-Castaneda, M.V.; Lee, M.-H.; Latres, E.; Polyak, K.; Lacombe, L.; Montgomery, K.; Mathew, S.; Krauter, K.; Sheinfeld, J.; Massague, J.; Cordon-Cardo, C. *Cancer Res.* 1995, 55, 1211-1214.
- (261) Pietenpol, J.A.; Bohlander, S.K.; Sato, Y.; Papadopoulos, N.; Liu, B.; Friedman, C.; Trask, B.J.; Roberts, J.M.; Kinzler, K.W.; Rowley, J.D.; Vogelstein, B. *Cancer Res.* 1995, 55, 1206-1210.
- (262) Slingerland, J.; Pagano, M. *J Cell Physiol.* 2000, 183, 10-17.
- (263) Naumann, U.; Weit, S.; Rieger, L.; Meyermann, R.; Weller, M. *Biochem.Biophys.Res Commun.* 1999, 261, 890-896.
- (264) Ishida, N.; Kitagawa, M.; Hatakeyama, S.; Nakayama, K. *J.Biol.Chem.* 2000, 275, 25146-25154.
- (265) Cobb, M.H. *Prog.Biophys.Mol Biol* 1999, 71, 479-500.
- (266) Maemura, M.; Iino, Y.; Koibuchi, Y.; Yokoe, T.; Morishita, Y. *Oncology* 1999, 57 Suppl 2, 37-44.
- (267) Nicholson, S.; Wright, C.; Sainsbury, J.R.; Halcrow, P.; Kelly, P.; Angus, B.; Farndon, J.R.; Harris, A.L. *Journal of Steroid Biochemistry and Molecular Biology* 1990, 37, 811-814.
- (268) Wright, C.; Angus, B.; Nicholson, S.; Sainsbury, J.R.; Cains, J.; Gullick, W.J.; Kelly, P.; Harris, A.L.; Horne, C.H. *Cancer Research* 1989, 49, 2087-2090.
- (269) Press, M.F.; Pike, M.C.; Chazin, V.R.; Hung, G.; Udove, J.A.; Markowicz, M.; Danyluk, J.; Godolphin, W.; Sliwowski, M.; Akita, R.e.al. *Can Res* 1993, 53, 4960-70.
- (270) Borg, A.; Baldetorp, B.; Ferno, M.; Killander, D.; Olsson, H.; Ryden, S.; Sigurdsson, H. *Cancer Lett* 1994, 81, 137-144.
- (271) Nicholson, S.; Sainsbury, J.R.; Halcrow, P.; Chambers, P.; Farndon, J.R.; Harris, A.L. *Lancet* 1989, 1, 182-185.
- (272) Houston, S.J.; Plunkett, T.A.; Barnes, D.M.; Smith, P.; Rubens, R.D.; Miles, D.W. *Br J Cancer* 1999, 79, 1220-1226.
- (273) Coutts, A.S.; Murphy, L.C. *Cancer Res* 1998, 58, 4071-4074.
- (274) El-Ashry, D.; Miller, D.; Kharbanda, S.; Lippman, M.E.; Kern, F.G. *Oncogene* 1997, 15, 435
- (275) Kurokawa, H.; Lenferink, A.E.; Simpson, J.f.; Pisacane, P.I.; Sliwowski, M.X.; Forbes, J.T.; Arteaga, C.L. *Cancer Res* 2000, 60, 5887-5894.
- (276) Fisher, B.; Constantino, J.; Redmond, C.; Poisson, R.; Bowman, D.; Couture, J.; Dimitrov, N.V.; Wolmark, N.; Wickerham, D.L.; Fisher, E.R.; Morgolese, R.; Robidoux, A.; Shibata, H.; Terz, J.; Paterson, A.H.G.; Feldman, M.I.; Farrar, W.; Evans, J.; Lickley, H.L.; Ketner, M. *N Engl J Med* 1989, 320, 479-84.
- (277) Novotny, L.; Rauko, P.; Vachalkova, A.; Peterson-Biggs, M. *Neoplasma* 2000, 47, 3-7.
- (278) Fuqua, S.A.; Chamness, G.C.; McGuire, W.L. *J Cell Biochem* 1993, 51, 135-139.
- (279) Osborne, C.K.; Coronado, E.; Allred, D.C.; Wiebe, V.; DeGregorio, M. *J Natl Cancer Inst* 1991, 83, 1477-1482.
- (280) Bronzert, D.A.; Greene, G.; Lippman, M.E. *Endocrinology* 1985, 117, 1409-1417.
- (281) Brunner, N.; Frandsen, T.L.; Holst-Hansen, C.; Bei, M.; Thompson, E.W.; Wakeling, A.E.; Lippman, M.E.; Clarke, R. *Cancer Res* 1993, 53, 3229-3232.
- (282) Lauper, N.; Beck, A.R.; Cariou, S.; Richman, L.; Hofmann, K.; Reith, W.; Slingerland, M.M.; Amati, B. *Oncogene* 1998, 17, 2637-2643.
- (283) Carroll, J.S.; Prall, O.W.; Musgrove, E.A.; Sutherland, R.L. *J Biol Chem* 2000, 275, 38221-38229.

- (284) Yang, H.-Y.; Zhou, B.P.; Hung, M.-C.; Lee, M.-H. *J.Biol.Chem.* **2000**, *275*, 24735-24739.
- (285) Radeva, G.; Petrocelli, T.; Behrend, E.; Leung-Hagesteijn, C.; Filmus, J.; Slingerland, J.; Dedhar, S. *J.Biol.Chem.* **1997**, *272*, 13937-13944.
- (286) Hori, M.; Inagawa, S.; Shimazaki, J.; Itabashi, M. *Pathol Res Pract* **2000**, *196*, 817-826.
- (287) Loda, M.; Cukor, B.; Tam, S.W.; Lavin, P.; Fiorentino, M.; Draetta, G.F.; Jessup, J.M.; Pagano, M. *Nature Med.* **1997**, *3*, 231-234.
- (288) Sebolt-Leopold, J.S.; Dudley, D.T.; Herrera, R.; Van Becelaere, K.; Wiland, A.; Gowan, R.C.; Tecle, H.; Barrett, S.D.; Bridges, A.; Przybranowski, S.; Leopold, W.R.; Saltiel, A.R. *Nat Med* **1999**, *5*, 810-816.
- (289) Massague, J. - *Annu.Rev.Biochem* **1998**, *67*, 753-91.
- (290) Reiss, M.; Barcellos-Hoff, M.H. - *Breast Cancer Res Treat* **1997**, *45*, 81-95.
- (291) Donovan, J.; Slingerland, J. *Breast Cancer Res.* **2000**, *2*, 116-124.
- (292) Reynisdottir, I.; Polyak, K.; Iavarone, A.; Massague, J. *Genes Dev.* **1995**, *9*, 1831-1845.
- (293) Ciarallo, S.; Subramaniam, V.; Hung, W.; Lee, J.H.; Kotchetkov, R.; Sandhu, C.; Milic, A.; Slingerland, J.M. - *Mol Cell Biol* **2002**, *22*, 2993-3002.
- (294) Lee, M.H.; Yang, H.Y. - *Cell Mol Life Sci.* **2001**, *58*, 1907-22.
- (295) Coats, S.; Whyte, P.; Fero, M.; Lacy, S.; Chung, G.; Randel, E.; Firpo, E.; Roberts, J.M. - *Curr.Biol* **1999**, *9*, 163-73.
- (296) Stampfer, M. *J.Tissue Cult.Methods* **1985**, *9*, 107-115.
- (297) Tait, L.; Soule, H.A.; Russo, J. - *Cancer Res* **1990**, *50*, 6087-94.
- (298) Dulic, V.; Lees, E.; Reed, S.I. *Science* **1992**, *257*, 1958-1961.
- (299) Amati, B.; Alevizopoulos, K.; Vlach, J. *Front.Biosci.* **1998**, *3*, D250-68, D250-D268
- (300) Feng, X.; Liang, Y.; Liang, M.; Zhai, W.; Lin, X. - *Mol Cell* **2002**, *9*, 133-43.
- (301) Staller, P.; Peukert, K.; Kiermaier, A.; Seoane, J.; Lukas, J.; Karsunky, H.; Moroy, T.; Bartek, J.; Massague, J.; Hanel, F.; Eilers, M. *Nat Cell Biol* **2001**, *3*, 392-399.
- (302) Jonuleit, T.; van der Kuip, H.; Miething, C.; Michels, H.; Hallek, M.; Duyster, J.; Aulitzky, W.E. - *Blood* **2000**, *96*, 1933-9.
- (303) Liu, X.; Sun, Y.; Ehrlich, M.; Lu, T.; Kloog, Y.; Weinberg, R.; Lodish, H.; Henis, Y.I. - *Oncogene* **2000**, *19*, 5926-35.
- (304) Liang, J.; Zubovits, J.; Petrocelli, T.; Kotchetkov, R.; Connor, M.; Han, K.; Lee J-H; Ciarallo, S.; Beniston R; Franssen, E.; Slingerland, J. *Submitted.* **2002**,
- (305) Mal, A.; Poon, R.; Howe, P.; Toyoshima, H.; Hunter, T.; Harter, M.L. - *Nature* **1996**, *380*, 262-5.
- (306) Collado, M.; Medema, R.; Garcia-Cao, I.; Dubuisson, M.; Barradas, M.; Glassford, J.; Rivas, C.; Burgering, B.M.F.A.; Serrano, M., - *J Biol Chem* **2000**, *275*, 21960-8.
- (307) Herzinger, T.; Wolf, D.; Eick, D.; Kind, P. - *Oncogene* **1995**, *10*, 2079-84.
- (308) Susini, T.; Baldi, F.; Howard, C.; Baldi, A.; Taddei, G.; Massi, D.; Rapi, S.; Savino, L.; Massi, G.; Giordano, A. - *J Clin.Oncol.* **1998**, *16*, 1085-93.
- (309) Tanaka, N.; Ogi, K.; Odajima, T.; Dehari, H.; Yamada, S.; Sonoda, T.; Kohama, G. - *Cancer* **2001**, *92*, 2117-25.
- (310) Massaro-Giordano, M.; Baldi, G.; De Luca, A.; Baldi, A.F.A.U.; Giordano, A. - *Clin.Cancer Res* **1999**, *5*, 1455-8.
- (311) Mateyak, M.; Obaya, A.J.; Sedivy, J.M. - *Mol Cell Biol* **1999**, *19*, 4672-83.

- (312) Queva, C.; McArthur, G Ramos, L.; Eisenman, R.N. - *Cell Growth Differ.*1999.*Dec.*;10,(12.):785.-96.
- (313) Cangi, M.; Cukor, B.; Soung, P.; Signoretti, S. Moreira, G.J.; Ranashinge, M.F; Cady, B.F. Pagano, M.; Loda, M. - *J Clin. Invest.*2000.*Sep.*;106,(6.):753.-61.

APPENDIX

The following manuscripts are attached:

Donovan JC, Milic A, Slingerland JM. Constitutive MEK/MAPK activation leads to p27Kip1 deregulation and antiestrogen resistance in human breast cancer cells. J Biol Chem. Nov 2001.

Donovan J, Slingerland J. Transforming growth factor-beta and breast cancer: Cell cycle arrest by transforming growth factor-beta and its disruption in cancer. Breast Cancer Res. 2000;2(2):116-24.

Cariou S, **Donovan JC***, Flanagan WM, Milic A, Bhattacharya N, Slingerland JM. Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells. Proc Natl Acad Sci U S A. 2000 Aug 1;97(16):9042-6.

(*co-first author)

Constitutive MEK/MAPK Activation Leads to p27^{Kip1} Deregulation and Antiestrogen Resistance in Human Breast Cancer Cells*

Received for publication, July 10, 2001
Published, JBC Papers in Press, August 29, 2001, DOI 10.1074/jbc.M106448200

Jeffrey C. H. Donovan, Andrea Milic, and Joyce M. Slingerland‡

From Molecular and Cell Biology, Sunnybrook and Women's College Health Science Centre,
Toronto, Ontario M4N 3M5, Canada

Antiestrogens, such as the drug tamoxifen, inhibit breast cancer growth by inducing cell cycle arrest. Antiestrogens require action of the cell cycle inhibitor p27^{Kip1} to mediate G₁ arrest in estrogen receptor-positive breast cancer cells. We report that constitutive activation of the mitogen-activated protein kinase (MAPK) pathway alters p27 phosphorylation, reduces p27 protein levels, reduces the cdk2 inhibitory activity of the remaining p27, and contributes to antiestrogen resistance. In two antiestrogen-resistant cell lines that showed increased MAPK activation, inhibition of the MAPK kinase (MEK) by addition of U0126 changed p27 phosphorylation and restored p27 inhibitory function and sensitivity to antiestrogens. Using antisense p27 oligonucleotides, we demonstrated that this restoration of antiestrogen-mediated cell cycle arrest required p27 function. These data suggest that oncogene-mediated MAPK activation, frequently observed in human breast cancers, contributes to antiestrogen resistance through p27 deregulation.

p27^{Kip1} is a member of the KIP¹ (kinase inhibitory protein) family of cdk inhibitors that regulate the cyclin-cdk complexes governing cell cycle transitions (1). The importance of p27 as a G₁-to-S phase regulator is highlighted by the finding that antisense-mediated inhibition of p27 expression is sufficient to induce cell cycle entry in quiescent fibroblasts (2) and in steroid-depleted breast cancer cells (3). p27 protein levels are high in G₀ and early G₁ during which time p27 binds tightly and inhibits cyclin E1-cdk2. p27 translation rates decrease, and its proteolysis increases during G₁-to-S phase progression, leading to p27 protein loss as cells enter S phase (4–6). p27 proteolysis is regulated by phosphorylation of p27 on threonine 187 (Thr-187) by cyclin E1-cdk2 (7, 8). While mutations or deletions in the p27 gene are uncommon (9, 10), p27 degradation is increased in many cancers, including breast cancer (11, 12).

* This work was supported in part by grants from the United States Army Department of Defense Breast Cancer Research Program (Ideas and Career Development to J. M. S. and Pre-doctoral Award to J. C. H. D.) and by Medical Research Council (MRC) of Canada funding (to A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Cancer Care Ontario and by the Burroughs Wellcome Fund. To whom correspondence should be addressed: Molecular and Cell Biology, Sunnybrook and Women's College Health Science Center, 2075 Bayview Ave., Toronto, Ontario M4N 3M5, Canada. Tel.: 416-480-6100 Ext. 3494; Fax: 416-480-5703; E-mail: joyce.slingerland@utoronto.ca.

¹ The abbreviations used are: KIP, kinase inhibitory protein; cdk, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ER, estrogen receptor; BrdUrd, bromodeoxyuridine; 2D-IEF, two-dimensional isoelectric focusing; AS, antisense.

An increasing body of data suggests that p27 is regulated by mitogenic signal transduction pathways, including Ras-dependent activation of the mitogen-activated protein kinase (MAPK) pathway (13–17). Many mitogens increase the cellular levels of GTP-bound Ras, leading to activation of the downstream target, Raf-1. The Raf-1 kinase can phosphorylate and activate the dual specificity kinases MEK1 and MEK2, which in turn activate MAPK (also known as p42^{ERK2} and p44^{ERK1}). Once activated, MAPK can phosphorylate several nuclear transcription factors including Myc, Elk, and Rsk (for review, see Ref. 18). p27 itself has several MAPK consensus sites, and MAPK can phosphorylate p27 *in vitro* (16) and reduce the ability of recombinant p27 to bind and inhibit cdk2 *in vitro* (15). While constitutive activation of Ras-MAPK can reduce p27 inhibitory function in immortal and cancer-derived lines, it is not clear whether MAPK directly regulates p27 during cell cycle progression in normal cell types.

Studies of p27 regulation by the Ras-MAPK pathway were initially carried out in fibroblasts (15, 19, 20). In NIH3T3 fibroblasts, Ras signaling is required for the down-regulation of p27 as cells approach the G₁-to-S phase transition (13, 20). Introduction of a dominant negative *ras* mutant prevented the loss of p27 in response to serum and inhibited S phase entry. Others have reported that Ras-MAPK activation reduces the ability of p27 to inhibit cdk2 through sequestration of p27 into cyclin D1-cdk4 complexes, rather than by promoting p27 protein loss (14).

Constitutive activation of the MAPK cascade may contribute to malignant progression of many human cancers (21). Although the causes of MAPK activation differ among tumors, in many cancers constitutive signaling from cell surface tyrosine kinase receptors contributes to activation of the Ras-Raf-1-MEK-MAPK pathway. For example, the epidermal growth factor receptor and HER2/c-ErbB-2, both of which activate the Ras-MAPK pathway, are overexpressed in up to 20 and 30% of breast cancers, respectively. Overexpression of these receptors has been associated with antiestrogen resistance and poor prognosis in primary breast cancers (22–27). Tissue culture models suggest that elevated MAPK activity may contribute to estrogen-independent growth of breast cancer cells (28–30).

Antiestrogen drugs, such as tamoxifen, are effective in the treatment and prevention of breast cancer (31–33). However, only two-thirds of estrogen receptor (ER)-positive breast cancers respond initially to antiestrogen therapy, and even sensitive tumors invariably acquire antiestrogen resistance (34). In most cases, acquired resistance is not due to a loss or mutation of the ER (35, 36). Numerous mechanisms have been proposed to explain the phenomenon of tamoxifen-resistant ER-positive breast cancer, including altered drug metabolism (37), altered binding of co-activator and co-repressor molecules to the antiestrogen-ER complex (38), and altered signal transduction

pathways that modulate ER activity (39) or regulate the cell cycle machinery (3).

The cell cycle inhibitor, p27^{Kip1}, is an essential mediator of cell cycle arrest by tamoxifen and other antiestrogenic drugs. We recently demonstrated that antisense-mediated down-regulation of p27^{Kip1} abrogated antiestrogen-induced cell cycle arrest in the ER-positive MCF-7 breast cancer line (3). p27 protein levels are frequently reduced in primary breast cancers compared with the normal breast epithelium, and low p27 protein levels are associated with poor prognosis and hormone independence (11, 40, 41). These observations stimulated the present study to investigate the relationships between Ras-MAPK pathway activation, antiestrogen resistance, and p27 function. Our data indicate that constitutive MEK activity alters p27 phosphorylation, reduces p27 inhibitory activity, and contributes to antiestrogen resistance in breast cancer.

EXPERIMENTAL PROCEDURES

Cell Culture.—MCF-7 cells (42) and LY-2 cells (43) were obtained from the laboratory of M. Lippman. The LCC2 line was a gift from G. Clarke (44). MCF-7 cells, stably transfected with full-length *HER2* cDNA (MCF-7/HER2-18), were kindly provided by C. Arteaga. All lines were grown in improved-modified essential medium (option Zn²⁺) supplemented with insulin and 10% fetal calf serum.

Flow Cytometric Analysis.—Cells were pulse-labeled with 10 μ M bromodeoxyuridine (BrdUrd) for 2 h and then fixed, stained with anti-BrdUrd-conjugated fluorescein isothiocyanate (Becton Dickinson) and counterstained with propidium iodide as described (45). Cell cycle analysis was carried out on a Becton Dickinson FACScan and Cell Quest Software. Values given for flow cytometric analysis represent the mean of repeat assays.

Cell Cycle Effects of Antiestrogens and MEK Inhibition.—For comparison of the effects of antiestrogens in MCF-7, MCF-7 transfectants, or LY-2 cell lines, cultures were treated by addition of 1 μ M 4-hydroxy-tamoxifen (4-OH-TAM) (Sigma) or 10 nM ICI 182780 (7 α -(4,4,5,5,5-pentafluoropentylsulfonyl) nonyl)estra-1,3,5, (10)-triene-3,17 β -diol, from Zeneca Pharmaceuticals) to complete medium, and samples were collected at 48 h thereafter for protein and flow cytometric analysis. The effects of MEK inhibition on the cell cycle were assayed following addition of 0.1 μ M U0126 (Promega) for 2 or 24 h prior to recovery for immunoblotting or flow cytometric analysis. The effects of MEK inhibition on antiestrogen sensitivity in the antiestrogen-resistant lines, LY-2 or MCF-7/HER2-18, were assayed by treating cells with 0.1 μ M U0126 for 2 h followed by an additional 48 h with either 1 μ M 4-OH-TAM or 10 nM ICI 182780 prior to recovery of cells for protein or flow cytometric analysis.

Antisense Oligonucleotide Transfections.—Antisense experiments were carried out as described (3). Phosphorothioate oligonucleotide sequences were as follows: GS5422 antisense p27 (ASp27) 5'-TG-GCTCTCXTGCGCC-3'; GS5585 mismatch p27 (MSMp27) 5'-TG-GCTCXCTTGCGCC-3'; X indicates the G-clamp modification of these oligonucleotides. The specificity of these oligonucleotides for p27 has been documented (2, 3). LY-2 cells were rendered quiescent by the addition of 0.1 μ M U0126 (Promega) for 2 h followed by antiestrogen treatment (10 nM ICI 182780, or 1 μ M 4-OH TAM) for a further 48 h. Quiescent cells were then transfected with 120 nM oligonucleotides using 2.5 μ g/ml cytofectin G3815 (Gilead Scientific, Foster City, California) for 6 h, followed by replacement with complete medium supplemented by U0126 and antiestrogen. Flow cytometry and proteins were analyzed prior to transfection and at 21 h thereafter.

Immunoblotting.—Cells lysis and immunoblotting were as described (45). Equal protein loading was verified by blotting for β -actin. To assay cyclin E1 complexes, cyclin E1 was immunoprecipitated from 200 μ g of protein lysate. Immunoprecipitates were resolved, transferred, and blotted with cyclin E1, cdk2, and p27 antibodies. Antibody alone controls were run along side immunoprecipitates.

Antibodies.—Monoclonal antibodies to p27 were obtained from Neomarkers (DCS-72) or Transduction Laboratories. p27 rabbit polyclonal serum (pAb5588) was provided by H. Toyoshima and T. Hunter (Salk Institute). Antibodies to p21 were from Santa Cruz, to cdk2 (PSTAIRE) from S. Reed (The Scripps Research Institute), to MEK, MAPK, and phospho-MAPK from New England Biolabs, to β -actin from Sigma, and to cyclin E1 (mAbs E12 and E172) from E. Harlow (Massachusetts General). These cyclin E1 antibodies are specific for cyclin E1(46). The ER antibody H222 was provided by Dr. G. Greene.

Cyclin-dependent Kinase Assays.—Cyclin E1 or p27 complexes were immunoprecipitated from 100 μ g of protein lysate and reacted with [γ -³²P]ATP and histone H1 as described (45). Radioactivity incorporated in histone substrate was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software. To determine background IgG-associated activity, nonspecific mouse IgG (for cyclin E1-cdk2 assays) or polyclonal rabbit IgG (for p27-associated kinase) immunoprecipitates from test lysates were collected on protein A, washed, and reacted with the kinase mixture as for cyclin E1 and p27 immunoprecipitates. Radioactivity incorporated in control nonspecific IgG immunoprecipitates was subtracted from test kinase values prior to graphing as in Refs. 45 and 47.

Production of Cyclin E1-cdk2 by Baculovirus Infection of Sf-9 Cells.—Sf-9 cells and TNM-FH media were obtained from Invitrogen. Adherent Sf-9 cells were co-infected with baculoviruses encoding human cyclin E1 or human cdk2 genes, and cyclin E1 and cdk2 were prepared as described (46). Sf-9 cell lysates containing cyclin E1 and cdk2 were used directly in p27 inhibitor assays.

Assays of p27 Inhibitory Function.—Cell lysates (250 μ g) from asynchronously proliferating MCF-7, MCF-7/MEK^{EE} (line M2), or LY-2 cells were immunoprecipitated with pAb5588 anti-p27 serum or control polyclonal rabbit IgG, and precipitates were collected on protein A-Sepharose beads. For testing heat-stable p27 inhibitor activity, p27 immunoprecipitates were washed, then boiled for 5 min in 200 μ l of reaction buffer, placed on ice, and then cleared by centrifugation. The p27 in the supernatant was recovered, and recombinant cyclin E1-cdk2 and dithiothreitol (1 mM final) were added and incubated at 30 °C for 30 min, followed by immunoprecipitation with either anti-cyclin E1 (mAbE172) or control polyclonal mouse IgG (Sigma). The un-inhibited control recombinant cyclin E1-cdk2 was treated with reaction buffer and incubated at 30 °C for 30 min without any added p27. Complexes were subsequently assayed for H1 kinase activity, and resulting activities were graphed as a % maximum un-inhibited cyclin E1-cdk2 activity.

Two-dimensional Isoelectric Focusing (2D-IEF) and Phosphatase Treatment.—Cells were lysed in ice-cold 0.1% Tween 20 lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 2.5 mM EGTA, pH 8.0, 10% glycerol, 10 mM β -glycerophosphate, 1 mM NaF, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na₂VO₄, 0.5 mM dithiothreitol, and 0.02 mg/ml each of aprotinin, leupeptin, and pepstatin). For 2D-IEF, p27 immunoprecipitates were denatured in 8 M urea, loaded onto immobilized non-linear pH gradient (pH 3–10) IEF strips and focused at 50,000 volt-hour using the IPGphor IEF system (Amersham Pharmacia Biotech). These assays yield highly reproducible electrophoretic resolution of isoforms because of the covalent linkage of the electrophoresis medium to a matrix. The IEF strip was equilibrated in 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS for 30 min before loading for SDS-polyacrylamide gel electrophoresis. Gels were transferred to polyvinylidene difluoride membrane, p27 isoforms were detected by immunoblotting using p27 antibody from Transduction Labs, and proteins were detected using enhanced chemiluminescence (ECL). For 2D-IEF of cyclin E1-bound p27, 3 mg of protein lysate was immunoprecipitated with the monoclonal mAb E172 antibody. Densitometric analysis of multiple ECL film exposures from repeat assays was used to determine the ratios of the various p27 isoforms. For phosphatase treatment, p27 immunoprecipitates were washed twice with phosphatase buffer (50 mM Tris, pH 8.0, 10% glycerol) and then incubated at 37 °C overnight with 66 units per 10 μ l of reaction of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals). To confirm that the most positively charged p27 isoforms represented unphosphorylated p27, cells were labeled with [³²P]orthophosphate (1 mCi/100-dish) for 3 h, and p27 immunoprecipitates were isolated and subjected to 2D-IEF. p27 immunoprecipitated from unlabeled cells was resolved by 2D-IEF in parallel with the labeled p27, and the resolution pattern of the cold p27 was compared with the phosphate-labeled p27.

RESULTS

p27 Deregulation in Antiestrogen-resistant LY-2.—We compared p27 levels in antiestrogen-sensitive MCF-7 (42) and the resistant MCF-7 derivative, LY-2 (43). The loss of antiestrogen responsiveness in LY-2 was not due to a loss of p27 protein (Fig. 1A).

The association of p27 with cyclin E1-cdk2 was assayed in asynchronously growing and antiestrogen-treated MCF-7 and LY-2 cells (Fig. 1B). Asynchronously proliferating LY-2 and MCF-7 cells had similar cell cycle profiles (Fig. 1B). When equal amounts of cyclin E1 were immunoprecipitated, the

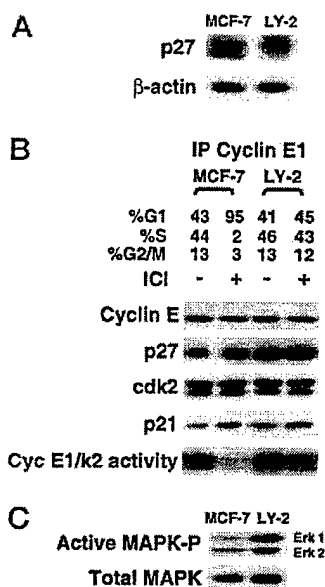


Fig. 1. Antiestrogen-resistant LY-2 show altered p27 regulation. A, asynchronously growing MCF-7 and LY-2 cell lysates were analyzed by Western blotting using the antibodies indicated. B, cyclin E1 immunoprecipitates (IP) from asynchronously proliferating and ICI 182780 (ICI)-treated cells were resolved and assayed for associated p27, p21, and cdk2 by immunoblotting or analyzed for associated histone H1 kinase activity as described under "Experimental Procedures." The cell cycle profiles from flow cytometric analysis are shown. C, lysates from asynchronously proliferating cells were analyzed for levels of total and active phospho-MAPK.

amount of p27 bound to cyclin E1-cdk2 in asynchronously growing LY-2 was nearly 4-fold higher than that in asynchronous MCF-7. There was no compensatory decrease in p21 binding to cyclin E1 in proliferating LY-2. Levels of cyclin E1-bound p21 were similar in proliferating MCF-7 and LY-2. Cyclin E1-bound cdk2 levels were similar in the two lines and were not affected by antiestrogens. Despite the increased p27 bound to cyclin E1-cdk2 in proliferating LY-2 cells, the histone H1 activity of these complexes was not reduced compared with cyclin E1-cdk2 from asynchronous MCF-7 (Fig. 1B). Antiestrogen treatment of MCF-7 with either ICI 182780 (ICI) or 4-OH-TAM (data shown here for ICI) caused a 3- to 5-fold increase in p27 binding to cyclin E1-cdk2, a 3-fold increase in cyclin E1-bound p21, inhibition of this kinase, and G₁ cell cycle arrest. Antiestrogen treatment of LY-2 caused a minimal increase in p21 binding, no change in the amount of p27 bound to cyclin E1-cdk2, no significant inhibition of cyclin E1-cdk2 activity, and no change in the cell cycle profile (Fig. 1B). These data suggested a functional alteration of p27 in LY-2 cells.

Since MAPK had been shown to alter p27 function in fibroblasts (15, 19, 20), we assayed MAPK activity by Western blotting using phospho-specific antibodies that detect activated MAPK. Although total MAPK protein levels were similar in LY-2 and MCF-7 cells, the levels of phosphorylated MAPK (both p42 ERK2 and p44 ERK1) were elevated nearly 8-fold in LY-2 (Fig. 1C). We also observed elevated MAPK activity in two other MCF-7 derived antiestrogen-resistant cell lines, LCC2 and MCF-7/HER2-18 (data not shown).

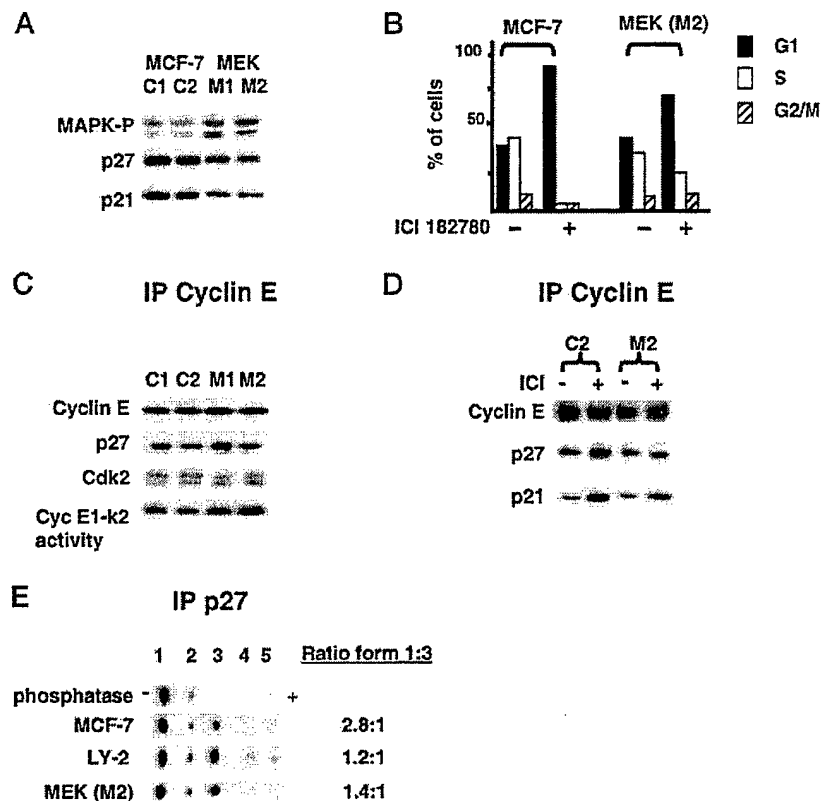
MEK1-transfected MCF-7 Lines Show Antiestrogen Resistance and Altered Binding of p27 to Cyclin E1-cdk2—To determine whether the increased MAPK activity observed in the antiestrogen-resistant lines was causally linked to the antiestrogen-resistant phenotype, we transfected MCF-7 cells with activated MEK1 (*MEK^{EE}*) or an active ERK2 allele (*ERK2-MEK*) and assayed stable transfected cell lines for antiestrogen

sensitivity. Transfectants showed MAPK activation compared with parental cells and empty vector controls (representative data shown for *MEK^{EE}* transfectants, labeled M1 and M2 in Fig. 2A). Of note, both p21 and p27 levels were reduced in *MEK^{EE}* transfectants (Fig. 2A). The level of the ER protein was not affected by the degree of MAPK activation achieved in these cells (data not shown). Asynchronously growing MAPK-activated lines and empty vector controls showed similar cell cycle profiles (Fig. 2B). Lines with constitutive MAPK activation showed partial resistance to 4-OH-TAM or ICI compared with the parental or vector alone controls (Fig. 2B).

Levels of cyclin E1-associated p21 and p27 were assayed in asynchronous *MEK^{EE}* transfectant lines and in the empty vector controls (Fig. 2C). Although densitometric analysis showed that total p27 levels in asynchronously growing *MEK^{EE}* transfectants were reduced by up to 3-fold compared with controls, the amount of p27 detected in cyclin E1-cdk2 complexes was not reduced (Fig. 2, C and D). Despite the similar amounts of both p27 and p21 bound to cyclin E1 in proliferating *MEK^{EE}* and control lines, equal amounts of cyclin E1-cdk2 showed approximately 2-fold higher kinase activity in *MEK^{EE}* transfectants compared with empty vector controls (Fig. 2, C and D). There was no change in cdk2 binding to cyclin E1, and the subtle increase in the proportion of the faster mobility, CAK-activated cdk2 bound to cyclin E1 would not suffice to mediate the 2-fold increase in cyclin E1-cdk2 activity in the *MEK^{EE}* lines (Fig. 2C). MCF-7 lines with constitutive MEK1 activation showed no increase in p27 binding to cyclin E1 following antiestrogens compared with that in parental MCF-7 or in the empty vector controls (representative data in Fig. 2D). The modest increase of p21 binding to cyclin E1-cdk2 may mediate the partial cell cycle inhibition after antiestrogen treatment of the M2 clone. Earlier work has established that increased KIP binding to cyclin E1-cdk2 in MCF-7 is essential for G₁ arrest by antiestrogens (3, 48). Thus, MAPK activation via *MEK^{EE}* or *ERK2-MEK* transfection may contribute to antiestrogen resistance, at least in part, by impairing the antiestrogen-mediated increase in p27 binding to cyclin E1-cdk2.

Altered p27 Phosphorylation in Antiestrogen-resistant Lines—The MAPK-activated transfectants and LY-2 cells show a number of similarities. Both showed more abundant p27 binding to cyclin E1-cdk2 in asynchronously proliferating cells than would have been predicted from the respective total cellular abundance of p27, and antiestrogens failed to cause an accumulation of p27 in cyclin E1-cdk2 complexes. We postulated that differences in p27 phosphorylation may be associated with these differences in p27 function. Under most one-dimensional SDS-polyacrylamide gel electrophoresis conditions, p27 does not show reproducible differences in gel mobility. 2D-IEF allowed resolution of different p27 phospho-isoforms that are not apparent on single dimension gel electrophoresis. 2D-IEF showed a reproducible difference between the phosphorylation profile of p27 in the antiestrogen-sensitive and -resistant lines. 2D-IEF of p27, using an amphoteric carrier with a non-linear pH range of 3–10, showed five p27 isoforms present in all three lines (labeled 1–5 in Fig. 2E). Form 1 migrates at the predicted isoelectric focusing point for p27 (pH = 6.54). Phosphatase treatment of the p27 immunoprecipitates confirmed that most of these different isoforms represent different phosphoforms of p27 (Fig. 2E). The minor amount of form 2 remaining after phosphatase treatment may reflect incomplete dephosphorylation. Alternatively, this may represent a hypophosphorylated form of p27 in which post-translational modification (e.g. myristylation) confers a more negative charge. When cells were [³²P]orthophosphate-labeled prior to p27 immunoprecipita-

FIG. 2. MAPK activation contributes to p27 deregulation and antiestrogen resistance. A, the levels of active phospho-MAPK, p27, and p21 were analyzed in two control cell lines transfected with empty vector (C1, C2) and in two MEK overexpressing MCF-7 clones (M1, M2). B, the cell cycle profiles of asynchronously proliferating and antiestrogen-treated MEK transfectants were compared with empty vector controls. C, cyclin E1-bound p27 and cdk2 and cyclin E1-associated kinase activities were assayed as in Fig. 1B. D, cyclin E1-bound p21 and p27 were assayed before (–) and after (+) ICI treatment. E, p27 immunoprecipitates from asynchronously proliferating MCF-7, LY-2, and MEK^{EE}-transfectant and (M2), were analyzed by 2D-IEF. The 2D-IEF of phosphatase-treated p27 from MCF-7 cells is shown in the upper panel. The different p27 isoforms were quantitated by densitometry.



tion and resolution by 2D-IEF, isoforms 1 and 2 were not radiolabeled, confirming their hypophosphorylated state (data not shown).

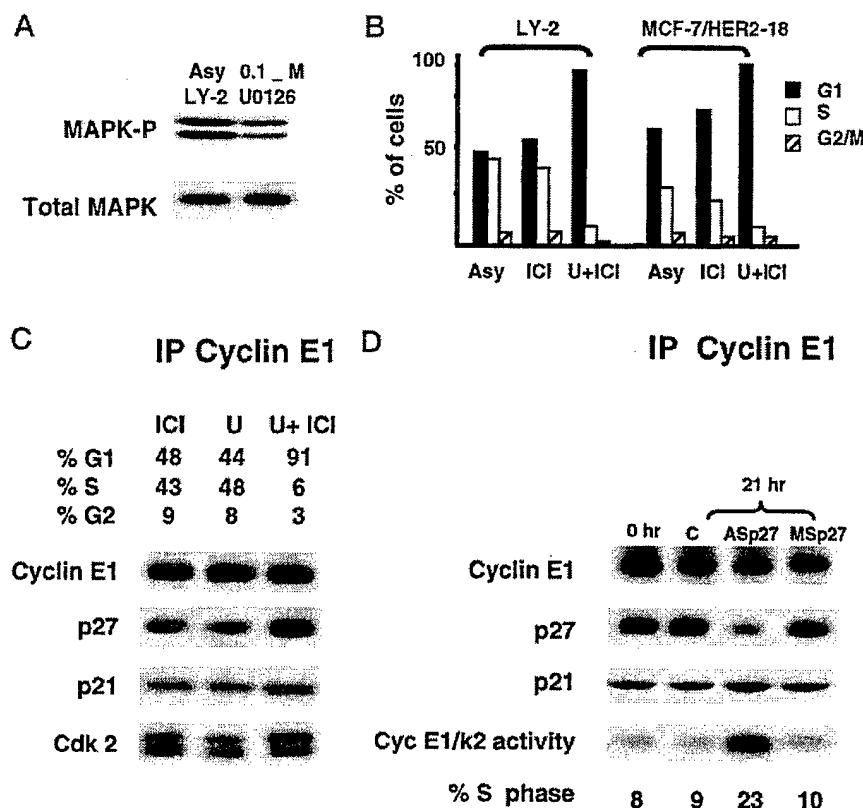
The relative abundance of the different isoforms of p27 differed between MCF-7 and the MAPK-activated lines, LY-2 and MCF-7/MEK^{EE}. In MCF-7, most of the p27 focused as isoform 1 with a lesser amount as isoform 3, and the ratio of these two forms (isoform 1:isoform 3) quantitated by densitometry was 3:1. The 2D-IEF patterns of p27 from LY-2 and the MEK transfectants were similar, and both differed from that seen in MCF-7. In these antiestrogen-resistant lines, form 3 showed greater relative abundance and the ratios of form 1 to form 3 were similar (~1:1 in MEK^{EE} clone M2 and LY-2, Fig. 2E). These observations support the notion that MEK/MAPK activation modulates p27 phosphorylation in these resistant cell lines.

MEK Inhibition Restores Sensitivity to Antiestrogen-mediated Cell Cycle Arrest—Treatment of the LY-2 line with 0.1 μ M of the MEK inhibitor, U0126, caused a 2.5-fold reduction of phospho-MAPK levels without affecting the total MAPK protein levels (Fig. 3A). Although this low dose of U0126 alone did not affect the cell cycle profile of the LY-2 cells, treatment with the combination of 0.1 μ M U0126 and either 1 μ M 4-OH-TAM or 10 nM ICI led to a G₁ arrest (data shown for ICI treatment, Fig. 3, B and C). MEK inhibition by 0.1 μ M U0126 also restored 4-OH-TAM or ICI-mediated G₁ arrest in the antiestrogen-resistant HER2/ErbB-2 overexpressing line, MCF-7/HER2-18 (Fig. 3B). The G₁ arrest following the combination of MEK inhibition and antiestrogen treatment in LY-2 (U+ICI) was accompanied by a 5-fold increase in the binding of p27 to cyclin E1-cdk2 complexes (Fig. 3C) and inhibition of cyclin E1-cdk2 activity (data not shown). p21 binding to cyclin E1-cdk2 was also modestly increased, and the proportion of CAK-phosphorylated cdk2 (faster mobility) bound to cyclin E1 was modestly reduced by the combined ICI 182780 and U0126 treatment.

The Arrest of LY-2 by MEK Inhibition and Antiestrogens Is p27-dependent—The increase in p27 association with cyclin E1-cdk2 in LY-2 cells treated by the combination of 0.1 μ M U0126 and 10 nM ICI was similar to that seen following antiestrogen treatment in the sensitive MCF-7 line (see Figs. 1B and 3C). We postulated that MAPK inhibition in LY-2 enhanced the cdk2 inhibitory function of p27 to facilitate cell cycle arrest by antiestrogens. If this were the case, then antisense-mediated inhibition of p27 expression in the U0126/ICI-treated cells should abrogate this drug-mediated arrest. U0126/ICI-arrested LY-2 cells were transfected with either antisense p27 (ASp27) oligonucleotides or mismatch control oligonucleotides (MSp27) or mock transfected with lipid only (control, C), and cells were recovered for flow cytometry and protein analysis at 21 h following completion of ASp27 transfection. The inhibition of p27 expression in ASp27-treated cells lead to cell cycle re-entry with ~23% cells in S phase at 21 h, in contrast to 8 and 9% of cells in S phase following lipid only (control, C, or mismatch (MSp27) transfection (Fig. 3D). The ASp27-mediated cell cycle re-entry was associated with loss of cyclin E1-bound p27 and cyclin E1-associated kinase activation (Fig. 3D). Control (lipid alone) and MSp27-transfected groups showed no cyclin E1-cdk2 activation. We also observed a similar result using the combination of 0.1 μ M U0126 and 1 μ M 4-OH-TAM (data not shown). Thus, in the LY-2 line, p27 became an essential mediator of G₁ arrest by antiestrogens following partial MEK/MAPK inhibition.

p27-immunoprecipitable Kinase Activity in Antiestrogen-treated LY-2 Cells—Proliferating LY-2 cells, with and without antiestrogen treatment, showed more abundant p27 association with active cyclin E1-cdk2 than was detected in inhibited cyclin E1-cdk2 complexes from antiestrogen-arrested MCF-7 cells (Fig. 1B). These data suggested impaired inhibitory function of cyclin E1-bound p27 in LY-2 cells. p27 immunoprecipitates were tested for associated histone H1 kinase activity in

FIG. 3. Inhibition of MAPK restores sensitivity to antiestrogens in LY-2. A, asynchronously growing LY-2 or LY-2 cells treated with 0.1 μ M U0126 were assayed for levels of total and active MAPK by immunoblotting. B, the cell cycle profile of LY-2 and MCF-7/HER2 cells were measured in asynchronously proliferating, ICI-treated and 0.1 μ M U0126 + ICI (U+ICI)-treated cells. C, LY-2 cells treated for 48 h with ICI, U0126 (U) or the combination of U0126 + ICI (U+ICI) and cell cycle profiles assayed by flow cytometry. Cyclin E1 immunoprecipitates were resolved and analyzed by immunoblotting with the indicated antibodies. D, LY-2 cells treated with the combination of U0126 + ICI were lysed before (0 h) or 21 h after transfection with lipid only (control group, C), ASp27 oligonucleotides (ASp27), or MSp27 oligonucleotides (MSp27). Cyclin E1 immunoprecipitates were resolved and associated proteins detected by immunoblotting. The % S phase cells and cyclin E1-associated kinase activities in each treatment group are shown.



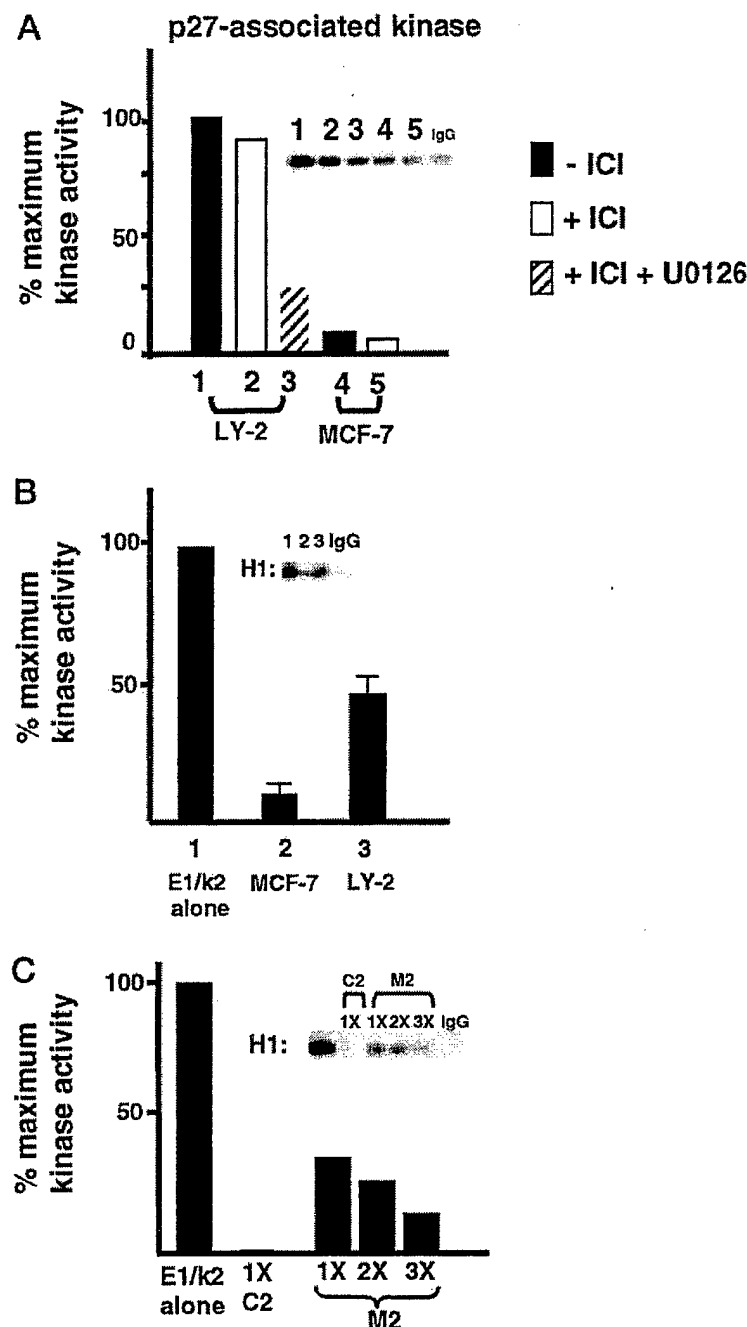
LY-2 and MCF-7 cells. Cdk2 complexes, but not cdk4 and 6 complexes, can use histone H1 as substrate. Histone H1 kinase activity was detected in p27 immunoprecipitates from asynchronous and ICI-treated LY-2 cells, but was negligible in asynchronous and ICI-treated MCF-7 when background activity in nonspecific antibody control immunoprecipitates was subtracted (Fig. 4A). The combination of 0.1 μ M U0126 and 10 nM ICI 182780 (U+ICI) inhibited the p27 immunoprecipitable kinase activity in LY-2 (Fig. 4A). Parallel p27 immunoprecipitates were resolved and blotted for associated cdk2, cyclin E1, and cyclin A. The amounts of cyclin and cdk2 bound to p27 in ICI-treated LY-2 and MCF-7 were similar, and there was no loss of p27-bound cyclin or cdk2 following ICI plus U0126 treatment LY-2 cells (data not shown).

MEK Activation Modulates p27 Inhibitory Function.—The inhibitory activity of p27 toward recombinant cyclin E1-cdk2 was compared in the MCF-7 and LY-2 lines (Fig. 4B), in the MCF-7/MEK-EE transfectant, M2, and in the empty vector control line, C2 (Fig. 4C). Equal amounts of p27 protein were immunoprecipitated from the indicated cell lines and boiled to release associated proteins, and then heat-stable p27 was tested for its ability to inhibit a fixed amount of recombinant cyclin E1-cdk2. The cyclin E1-cdk2 complexes were then immunoprecipitated, and kinase activity was assayed. The activity of the p27-treated cyclin E1-cdk2 was expressed as a % of control, uninhibited cyclin E1-cdk2. p27 from the MCF-7 line had approximately four times the inhibitory potency as p27 from the LY-2 line (Fig. 4B). Similarly, MEK overexpression in the M2 line impaired the inhibitory function of p27 (Fig. 4C). The cyclin E1-cdk2 inhibitory activity of increasing amounts of p27 from the MEK^{EE}-transfected M2 line was compared with that of p27 from the vector alone control, C2. Even a 3-fold (3 \times) excess of p27 in the M2 line did not achieve the same level of cyclin E1-cdk2 inhibition shown by p27 (1 \times) from the control line.

MEK Inhibition Modulates p27 Phosphorylation.—Our antisense experiments showed that p27 is essential for the antiestrogen arrest of LY-2 following partial MEK inhibition (Fig. 3D). Since MEK inhibition restored antiestrogen arrest, we postulated that MEK inhibition might alter p27 phosphorylation. As seen in asynchronously proliferating cells (Fig. 2E), the 2D-IEF of p27 from antiestrogen-treated MCF-7 and LY-2 cells showed five distinct p27 isoforms (labeled 1–5 in Fig. 5A) with isoforms 1 and 3 again being the most abundant. p27 from antiestrogen-arrested MCF-7 showed a predominance of isoform 1, with the ratio of isoforms 1 to 3 being 2:1. In antiestrogen-treated LY-2 cells, form 3 was the predominant form, with the isoform 1:isoform 3 ratio at 1:2. Treatment with 0.1 μ M U0126 together with either 4-OH-TAM or ICI changed the p27 phosphorylation profile in LY-2 cells to one that more closely resembled that in antiestrogen-arrested MCF-7 cells, with the p27 isoform 1 more abundant than isoform 3 at a ratio of 2:1 (data shown for ICI treatment in Fig. 5A). In all cell types, ICI treatment increased the relative abundance of isoforms 4 and 5 compared with that of untreated cells.

We tested whether the changes in total cellular p27 phosphorylation were reflected by changes in the phosphorylation of cyclin E1-bound p27 (Fig. 5B). Cyclin E1-bound p27 in the ICI-treated LY-2 line showed a predominance of isoform 3 (the ratio of isoform 1:isoform 3 was 1:6 by densitometry), whereas LY-2 cells arrested by the combination of MEK inhibition and antiestrogen showed a cyclin E1-associated p27 phosphorylation pattern more closely resembling that in antiestrogen-arrested MCF-7 (isoform 1:isoform 3 ratio nearly 1:1 in both). These data suggest that the combination of MEK inhibition and antiestrogen treatment may restore the cyclin E1-cdk2 inhibitory function of p27 in LY-2, at least in part, by altering p27 phosphorylation.

FIG. 4. MEK inhibition partially restores p27 inhibitory function and changes the pattern of p27 phosphorylation. A, equal amounts of p27 were precipitated from asynchronous (lanes 1, 4) and ICI-treated LY-2 and MCF-7 cells (lanes 2, 5) and from LY-2 cells treated with both U0126 + ICI (lane 3) and analyzed for associated histone H1 kinase activity. Radioactivity in the histone H1 substrate was quantitated by Phosphor-Imager and expressed as a percentage of maximum activity after subtraction of background from IgG control (lane 6) and graphed. The inset shows the autoradiogram of activity in histone H1. The data presented are the mean of repeat experiments. B, p27 was immunoprecipitated from asynchronously proliferating MCF-7 and LY-2 lysates and boiled, and equal amounts of p27 were added to recombinant cyclin E1-cdk2. Cyclin E1 complexes were then immunoprecipitated and assayed for histone H1 (H1) kinase activity. Inhibition of cyclin E1-cdk2 activity by added p27 is shown. Radioactivity incorporated into the histone H1 substrate is shown in the autoradiograph (see inset, upper right) and graphed as % maximum uninhibited cyclin E1-cdk2 activity. Mouse IgG served as an immunoprecipitation control. C, equal amounts of p27 were immunoprecipitated from the asynchronously proliferating MCF-7/MEK^{EE} clone (M2) or from the empty vector control (C2) and cyclin E1-cdk2 inhibitor assayed as in B.



DISCUSSION

The key roles of p21 and p27 in antiestrogen arrest have been demonstrated in earlier studies (3, 48). Antiestrogens increase cyclin E1-cdk2-KIP binding, and immunodepletion of p21 and p27 from steroid-depleted or tamoxifen-arrested cells removes essentially all cellular cyclin E1-cdk2 (49–51), suggesting that these cyclin complexes are fully saturated by p21 or p27 in arrested cells. Recent work with antisense (AS) p27 and p21 demonstrated that inhibition of expression of either KIP from antiestrogen-arrested cells leads to cell cycle re-entry (3, 48). In addition to increased KIP-cdk binding, other cell cycle effectors contribute to G₁ arrest by antiestrogens. These include reductions in c-Myc and cyclin D1 and Cdc25A, increased p15, and potentially, the accumulation of cdk2 in a non-CAK-activated form (3, 50–53). However, while induction

of cell cycle arrest by antiestrogens has multiple effectors, the antisense studies demonstrate that KIP function is required for maintenance of arrest. Moreover, the present work indicates that deregulation of p27 inhibits antiestrogen responsiveness.

Our data suggest that constitutive MEK/MAPK activation contributes to the development of antiestrogen resistance in ER-positive breast cancer cells, at least in part, by compromising the inhibitory function of p27. We show here that a non-cytostatic and non-cytotoxic dose of the MEK inhibitor, U0126, restored sensitivity to G₁ arrest by antiestrogens in the widely used LY-2 model of antiestrogen resistance. Moreover, transfection of *HER2* or *MEK^{EE}* into MCF-7 impaired antiestrogen responses. In antiestrogen-treated LY-2 and MCF-7/MEK^{EE} transfectants, p27 failed to accumulate in cyclin E1-cdk2 complexes and did not inhibit this kinase. MEK inhibition by

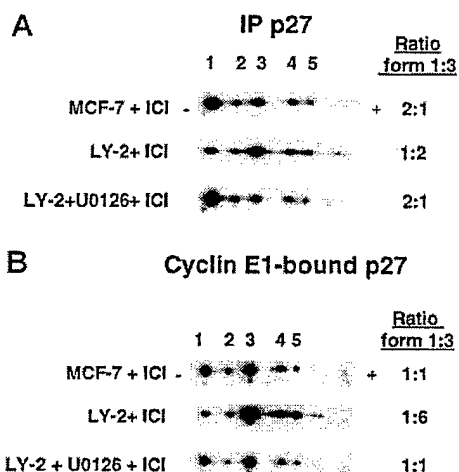


FIG. 5. MEK inhibition modulates p27 phosphorylation. A and B, the 2D-IEF patterns of immunoprecipitated p27 (A) or cyclin E1-bound p27 (B) were assayed in MCF-7 and LY-2 cells after 48 h of treatment with ICI, and in LY-2 cells after 48 h treatment with both ICI and U0126.

U0126 in these antiestrogen-resistant lines altered p27 phosphorylation and restored the inhibitory binding of p27 to cdk2 following antiestrogen treatment. Thus, MEK/MAPK-dependant p27 phosphorylation events are associated with a reduced ability to inhibit cdk2.

Through the course of selection of the antiestrogen-resistant LY-2, p27 regulation has been altered such that its binding to cyclin E1-cdk2 is increased without a commensurate reduction in cyclin E1-cdk2 activity. In antiestrogen-mediated arrest of MCF-7, a 3-fold increase in p27 binding to cyclin E1-cdk2 is sufficient for cdk2 inactivation and cell cycle arrest (3). The approximately 4-fold increase in cyclin E1-bound p27 in asynchronously growing LY-2 cells relative to that in proliferating MCF-7 and the failure of antiestrogens to increase p27 binding to cyclin E1-cdk2 in LY-2 prompted further investigation of p27 function in these lines. Indeed, both cyclin E1 and p27 immunoprecipitates contain detectable histone H1 kinase activity in LY-2 cells. While the p27-associated kinase activity could reflect dissociation of p27 from cyclin E1-cdk2 *in vitro* following immunoprecipitation of the complexes, the increased binding of p27 to cyclin E1 without loss of kinase activity in asynchronous LY-2 suggests that some of the cyclin E1-cdk2-p27 complexes may retain activity *in vivo*. Detection of p27-immunoprecipitable kinase activity has been reported by others (54, 55). The elevated level of p27 protein in the LY-2 line may reflect MAPK-independent events that have occurred throughout the course of selection of this line (44).

p27 levels were reduced in the MEK^{EE} transfectants, consistent with the observation by others that Ras-MAPK contributes to p27 degradation (15, 20, 56). Despite the lower total p27 protein levels in these cells, cyclin E1-bound p27 levels were not reduced. Moreover, while cyclin E1-bound p21 and p27 levels were similar in MEK^{EE} transfectants and control lines, cyclin E1-cdk2 activity was increased in asynchronously proliferating MEK^{EE} transfectants compared with controls. Thus, MAPK activation at the levels achieved here, may favor the association of p27 with cdk2 in a poorly inhibitory form, such that some of the cyclin E1-cdk2-p27 complexes retain activity. This effect may be separable from the effect of MAPK on p27 stability. p27 forms that can bind cyclin E1-cdk2 but fail to inhibit this kinase have been modeled previously *in vitro* (8). Further evidence to support functional alteration of p27 in the LY-2 and MEK^{EE} transfectant MCF-7 lines is provided by as-

says of p27 inhibitory function. p27 from both LY-2 and the MEK^{EE}-transfected line, M2, both showed a reduced ability to inhibit recombinant cyclin E1-cdk2 *in vitro*. As an alternate model, MEK/MAPK activation may reduce the stability with which p27 binds to cyclin E1-cdk2 *in vitro*, allowing detection of this kinase activity in p27 immunoprecipitates through dissociation *in vitro*. A reduced ability to bind cyclin E1-cdk2 could also account for the reduced inhibitory activity of heat-stable p27 isolated from the resistant lines.

Signal transduction pathways have been shown to affect p27 inhibitory function (15, 57), raising the possibility that phosphorylation events may modulate p27 function. Overexpression of the integrin-linked kinase causes a reduction in inhibitory activity of p27 toward cyclin E1-cdk2 (57). Here we showed that the antiestrogen ICI182780 modulates p27 phosphorylation in MCF-7 cells, increasing the relative amounts of p27 isoforms 3, 5, and 6. We also showed an association between altered p27 inhibitory function and altered phosphorylation in LY-2 and MCF-7/MEK^{EE} cells, suggesting that deregulated p27 phosphorylation may be causally linked to antiestrogen resistance. Although MAPK can phosphorylate p27 *in vitro* (15–17), it is not known at present whether direct phosphorylation of p27 by MAPK occurs *in vivo*. The effects of MAPK on the p27 phosphorylation profile may be indirect. p27 contains several potential MAPK consensus sites, including serine 10 (Ser-10), Ser-178, and Thr-187. Ser-10 has recently been shown to be a major p27 phosphorylation site in G₀-arrested cells, although it may not be a physiological MAPK target site (16). Since a p27 mutation converting Ser-10 to alanine or aspartate did not affect the ability of p27 to inhibit cyclin E1-cdk2 *in vitro* (16), the MAPK-dependent pathway that modulates both p27 phosphorylation and its ability to inhibit cdk2 cannot uniquely affect Ser-10. Moreover, the phosphorylation of p27 at Thr-187 that regulates its recognition by the F box protein Skp-2, does not affect the cdk2 inhibitory function of p27 (8). Thus, phosphorylation at sites other than Ser-10 and Thr-187 may be required for the MEK/MAPK-dependent phosphorylation of p27 that modulates its cdk2 inhibitory function. The identity of the different 2D-IEF phospho-isoforms of p27 observed by 2D-IEF warrants further investigation.

The causes of MAPK activation in human cancers differ among different tumors. MAPK activation is increased in up to 50% of breast cancers compared with normal breast epithelium and is associated with poor patient prognosis (58–60). HER-2/erbB-2 overexpression, seen in up to 30% of breast cancers is often associated with antiestrogen resistance (27). HER-2/erbB-2 signaling has been shown to decrease p27 stability via MAPK activation (56). In the HER-2 overexpressing MCF-7/HER-2-18, MEK inhibition by U0126 restored sensitivity to antiestrogens. Taken together, the present study links HER-2/erbB-2 activation and antiestrogen resistance through MAPK-dependent alterations in p27 function.

In addition to its mechanistic relevance to breast cancer, the observed link between p27 dysfunction and MAPK activation has implications for many types of cancers. The reduced levels of p27 observed in many cancers (colon, lung, prostate, gastric) may reflect oncogenic activation of the Ras/MEK/MAPK pathway (12). For example, the increased p27 proteolytic activity observed in colon cancer lysates may result from oncogenic activation of K-Ras in these cancers (61). There is a strong molecular rationale supporting the continued development of MEK/MAPK inhibitory drugs. A number of MEK inhibitors have shown good oral bioavailability and efficacy in preclinical trials (62). Tumor-specific MEK inhibitors may have the potential to restore p27 protein levels and inhibitory function and thereby restrain tumor growth.

Acknowledgments—We thank Drs. T. Hunter and H. Toyoshima for the pAb5588 p27 antibody, Dr. G. Greene for the ER (H222) antibody, Dr. D. Templeton and Dr. M. Cobb for the EE-CMV MEK2A and ERK2MEK1 plasmids, respectively, and Dr. M. Flanagan and Gilead Sciences for providing the p27 oligonucleotides. We also thank Drs. W. Hung, V. Subramaniam and J.-H. Lee for expertise in 2D-IEF and Dr. J. Liang for useful discussions regarding MEK inhibitors.

REFERENCES

- Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* **13**, 1501–1512
- Coats, S., Flanagan, M., Nourse, J., and Roberts, J. M. (1996) *Science* **272**, 877–880
- Cariou, S., Donovan, J., Flanagan, W., Milic, A., Bhattacharya, N., and Slingerland, J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 9042–9046
- Hengst, L., and Reed, S. I. (1996) *Science* **271**, 1861–1864
- Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. (1995) *Science* **269**, 682–685
- Millard, S. S., Yan, J. S., Nguyen, H., Pagano, M., Kiyokawa, H., and Koff, A. (1997) *J. Biol. Chem.* **272**, 7093–7098
- Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Churman, B. E. (1997) *Genes Dev.* **11**, 1464–1478
- Vlach, J., Hennecke, S., and Amati, B. (1997) *EMBO J.* **16**, 5334–5344
- Ponce-Castaneda, M. V., Lee, M.-H., Latres, E., Polyak, K., Lacombe, L., Montgomery, K., Mathew, S., Krauter, K., Sheinfeld, J., Massague, J., and Cordon-Cardo, C. (1995) *Cancer Res.* **55**, 1211–1214
- Pietenpol, J. A., Bohlender, S. K., Sato, Y., Papadopoulos, N., Liu, B., Friedman, C., Trask, B. J., Roberts, J. M., Kinzler, K. W., Rowley, J. D., and Vogelstein, B. (1995) *Cancer Res.* **55**, 1206–1210
- Tan, P., Cady, B., Wanner, M., Worland, P., Cukor, B., Magi-Galluzzi, C., Lavin, P., Draetta, G., Pagano, M., and Loda, M. (1997) *Cancer Res.* **57**, 1259–1263
- Slingerland, J., and Pagano, M. (2000) *J. Cell. Physiol.* **183**, 10–17
- Akts, H., Cai, H., and Cooper, G. M. (1997) *Mol. Biol. Cell* **17**, 3850–3857
- Cheng, M., Sexl, V., Sherr, C. J., and Roussel, M. F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 1091–1096
- Kawada, M., Yamagoe, S., Murakami, Y., Suzuki, K., Mizuno, S., and Uehara, Y. (1997) *Oncogene* **15**, 629–637
- Naumann, U., Weit, S., Rieger, L., Meyermann, R., and Weller, M. (1999) *Biochem. Biophys. Res. Commun.* **261**, 890–896
- Ishida, N., Kitagawa, M., Hatakeyama, S., and Nakayama, K. (2000) *J. Biol. Chem.* **275**, 25146–25154
- Cobb, M. H. (1999) *Prog. Biophys. Mol. Biol.* **71**, 479–500
- Leone, G., DeGregori, J., Sears, R., Jakoi, L., and Nevins, J. R. (1997) *Nature* **387**, 422–426
- Takuwa, N., and Takuwa, Y. (1997) *Mol. Cell. Biol.* **17**, 5348–5358
- Maemura, M., Iino, Y., Koibuchi, Y., Yokoe, T., and Morishita, Y. (1999) *Oncology* **57** Suppl. 2, 37–44
- Nicholson, S., Wright, C., Sainsbury, J. R., Halcrow, P., Kelly, P., Angus, B., Farndon, J. R., and Harris, A. L. (1990) *J. Steroid Biochem. Mol. Biol.* **37**, 811–814
- Wright, C., Angus, B., Nicholson, S., Sainsbury, J. R., Cains, J., Gullick, W. J., Kelly, P., Harris, A. L., and Horne, C. H. (1989) *Cancer Res.* **49**, 2087–2090
- Press, M. F., Pike, M. C., Chazin, V. R., Hung, G., Udove, J. A., Markowicz, M., Danyluk, J., Godolphin, W., Sliwkowski, M., and Akita, R., et al. (1993) *Cancer Res.* **53**, 4960–4970
- Borg, A., Baldestorp, B., Ferno, M., Killander, D., Olsson, H., Ryden, S., and Sigurdsson, H. (1994) *Cancer Lett.* **81**, 137–144
- Nicholson, S., Sainsbury, J. R., Halcrow, P., Chambers, P., Farndon, J. R., and Harris, A. L. (1989) *Lancet* **1**, 182–185
- Houston, S. J., Plunkett, T. A., Barnes, D. M., Smith, P., Rubens, R. D., and Miles, D. W. (1999) *Br. J. Cancer* **79**, 1220–1226
- Coutts, A. S., and Murphy, L. C. (1998) *Cancer Res.* **58**, 4071–4074
- El-Ashry, D., Miller, D., Kharbanda, S., Lippman, M. E., and Kern, F. G. (1997) *Oncogene* **15**, 435
- Kurokawa, H., Lenferink, A. E., Simpson, J. F., Pisacane, P. I., Sliwkowski, M. X., Forbes, J. T., and Arteaga, C. L. (2000) *Cancer Res.* **60**, 5887–5894
- Fisher, B., Constantino, J., Redmond, C., Poisson, R., Bowman, D., Couture, J., Dimitrov, N. V., Wolmark, N., Wickerham, D. L., Fisher, E. R., Morgolesse, R., Robidoux, A., Shibata, H., Terz, J., Paterson, A. H. G., Feldman, M. I., Farrar, W., Evans, J., Lickley, H. L., and Ketner, M. (1989) *N. Engl. J. Med.* **320**, 479–484
- Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., Daly, M., Wieand, S., Tan-Chiu, E., Ford, L., and Wolmark, N. (1998) *J. Natl. Cancer Inst.* **90**, 1371–1388
- Novotny, L., Rauko, P., Vachalkova, A., and Peterson-Biggs, M. (2000) *Neoplasma* **47**, 3–7
- Jordan, V. C. (1995) *Breast Cancer Res. Treat.* **36**, 267–285
- Robertson, J. F. R. (1996) *Br. J. Cancer* **73**, 5–12
- Fuqua, S. A., Chamness, G. C., and McGuire, W. L. (1993) *J. Cell. Biochem.* **51**, 135–139
- Osborne, C. K., Coronado, E., Allred, D. C., Wiebe, V., and DeGregorio, M. (1991) *J. Natl. Cancer Inst.* **83**, 1477–1482
- Takimoto, G. S., Graham, J. D., Jackson, T. A., Tung, L., Powell, R. L., Horwitz, L. D., and Horwitz, K. B. (1999) *J. Steroid Biochem. Mol. Biol.* **69**, 45–50
- Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masuhige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* **270**, 1491–1494
- Catzavelos, C., Bhattacharya, N., Ung, Y. C., Wilson, J. A., Roncari, L., Sandhu, C., Shaw, P., Yeger, H., Morava-Protzner, I., Kapusta, L., Franssen, E., Pritchard, K. I., and Slingerland, J. M. (1997) *Nature Med.* **3**, 227–230
- Porter, P. L., Malone, K. E., Heagerty, P. J., Alexander, G. M., Gatti, L. A., Firpo, E. J., Daling, J. R., and Roberts, J. M. (1997) *Nature Med.* **3**, 222–225
- Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brennan, S. (1973) *J. Natl. Cancer Inst.* **51**, 1409–1413
- Bronzert, D. A., Greene, G., and Lippman, M. E. (1985) *Endocrinology* **117**, 1409–1417
- Brunner, N., Frandsen, T. L., Holst-Hansen, C., Bei, M., Thompson, E. W., Wakeling, A. E., Lippman, M. E., and Clarke, R. (1993) *Cancer Res.* **53**, 3229–3232
- Sandhu, C., Garbe, J., Daksis, J., Pan, C.-H., Bhattacharya, N., Yaswen, P., Koh, J., Slingerland, J., and Stampfer, M. R. (1997) *Mol. Cell. Biol.* **17**, 2458–2467
- Lauper, N., Beck, A. R., Cariou, S., Richman, L., Hofmann, K., Reith, W., Slingerland, M. M., and Amati, B. (1998) *Oncogene* **17**, 2637–2643
- Slingerland, J. M., Hengst, L., Pan, C.-H., Alexander, D., Stampfer, M. R., and Reed, S. I. (1994) *Mol. Cell. Biol.* **14**, 3683–3694
- Carroll, J. S., Prall, O. W., Musgrove, E. A., and Sutherland, R. L. (2000) *J. Biol. Chem.* **275**, 38221–38229
- Foster, J., and Wimalasen, J. (1996) *Mol. Endocrinol.* **0**, 488–496
- Prall, O. W. J., Sarcevic, B., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (1997) *J. Biol. Chem.* **272**, 10882–10894
- Planas-Silva, M. D., and Weinberg, R. A. (1997) *Mol. Cell. Biol.* **17**, 4059–4069
- Prall, O. W. J., Rogan, E. M., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (1998) *Mol. Cell. Biol.* **18**, 4499–4508
- Foster, J. S., Henley, D. C., Bukovsky, A., Seth, P., and Wimalasena, J. (2001) *Mol. Cell., Biol.* **21**, 794–810
- LaBaer, J., Garret, M., Steenson, M., Slingerland, J., Sandhu, C., Chou, H., Fattaey, A., and Harlow, H. (1997) *Genes Dev.* **11**, 847–862
- Florenes, V. A., Bhattacharya, N., Bani, M. R., Ben-David, J., Kerbel, R. S., and Slingerland, J. M. (1996) *Oncogene* **13**, 2447–2457
- Yang, H.-Y., Zhou, B. P., Hung, M.-C., and Lee, M.-H. (2000) *J. Biol. Chem.* **275**, 24735–24739
- Radeva, G., Petrocelli, T., Behrend, E., Leung-Hageteijn, C., Filmus, J., Slingerland, J., and Dedhar, S. (1997) *J. Biol. Chem.* **272**, 13937–13944
- Hori, M., Inagawa, S., Shimazaki, J., and Itabashi, M. (2000) *Pathol. Res. Pract.* **196**, 817–826
- Mueller, H., Flury, N., Eppenberger-Castori, S., Kueng, W., David, F., and Eppenberger, U. (2000) *Int. J. Cancer* **89**, 384–388
- Salh, B., Marotta, A., Matthewson, C., Ahluwalia, M., Flint, J., Owen, D., and Pelech, S. (1999) *Anticancer Res.* **19**, 731–740
- Loda, M., Cukor, B., Tam, S. W., Lavin, P., Fiorentino, M., Draetta, G. F., Jessup, J. M., and Pagano, M. (1997) *Nat. Med.* **3**, 231–234
- Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Tecle, H., Barrett, S. D., Bridges, A., Przybranowski, S., Leopold, W. R., and Saltiel, A. R. (1999) *Nat. Med.* **5**, 810–816

Review

Transforming growth factor- β and breast cancer Cell cycle arrest by transforming growth factor- β and its disruption in cancer

Jeffrey Donovan and Joyce Slingerland

University of Toronto, and Toronto Sunnybrook Regional Cancer Centre and Sunnybrook and Women's College Health Sciences Centre, Toronto, Ontario, Canada

Received: 7 February 2000

Accepted: 7 February 2000

Published: 21 February 2000

Breast Cancer Res 2000, 2:116-124

© Current Science Ltd

Abstract

Altered responsiveness to extracellular signals and cell cycle dysregulation are hallmarks of cancer. The cell cycle is governed by cyclin-dependent kinases (cdks) that integrate mitogenic and growth inhibitory signals. Transforming growth factor (TGF)- β mediates G₁ cell cycle arrest by inducing or activating cdk inhibitors, and by inhibiting factors required for cdk activation. Mechanisms that lead to cell cycle arrest by TGF- β are reviewed. Loss of growth inhibition by TGF- β occurs early in breast cell transformation, and may contribute to breast cancer progression. Dysregulation of cell cycle effectors at many different levels may contribute to loss of G₁ arrest by TGF- β . Elucidation of these pathways in breast cancer may ultimately lead to novel and more effective treatments for this disease.

Keywords: breast cancer, cell cycle, cyclin-dependent kinase inhibitor, human mammary epithelial cells, transforming growth factor- β

Introduction

TGF- β is a potent inhibitor of mammary epithelial cell proliferation [1,2] and regulates mammary development *in vivo* [3-5]. Mammary-specific overexpression of TGF- β in transgenic mice can induce mammary hypoplasia and inhibit tumorigenesis [6-8]. Although normal human mammary epithelial cells (HMECs) are exquisitely sensitive to TGF- β [9], human breast cancer lines require 10-fold to 100-fold more TGF- β to produce an antimitogenic response, and some show complete loss of this effect [10].

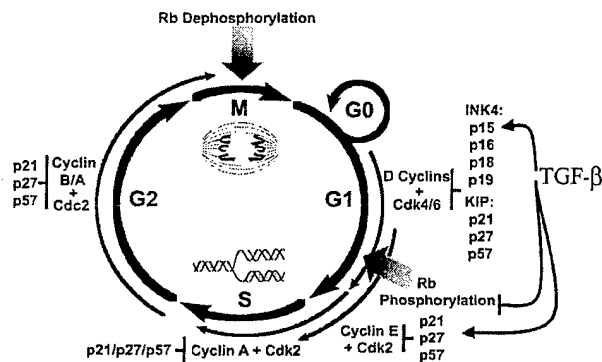
Although loss of growth inhibition by TGF- β in human cancers can arise through loss of TGF- β production or through mutational inactivation of the TGF- β receptors and Smad signalling molecules [11,12], these defects are not

observed in most arrest-resistant cancer lines. This observation, and the frequent appearance of resistance to more than one inhibitory cytokine in human tumours [13] emphasize the importance of the cell cycle effectors of growth arrest induced by TGF- β as targets for inactivation in cancer.

TGF- β can either lengthen G₁ transit time or cause arrest in late G₁ phase [14]. This cell cycle arrest is usually reversible [15,16], but in some cases is associated with terminal differentiation [17,18,19]. Because TGF- β arrests susceptible cells in the G₁ phase, a brief review of cell cycle regulation is presented. This is followed by a review of the multiple and often, complementary mechanisms that contributing to G₁ phase arrest by TGF- β and of how they are disrupted in breast and other cancers.

Cdc = cell division cycle; cdk = cyclin-dependent kinase; CAK = cdk-activating kinase; EGF = epidermal growth factor; HMEC = human mammary epithelial cell; INK4 = inhibitor of cdk4; KIP = kinase inhibitor protein; pRb = retinoblastoma protein; TGF = transforming growth factor.

Figure 1



The cell cycle. Cell cycle progression is governed by cyclin-dependent kinases (cdks), the activities of which are regulated by binding of cyclins, by phosphorylation and by the cdk inhibitors [the inhibitor of cdk4 (INK4) family: p15, p16, p18 and p19; and the kinase inhibitor protein (KIP) family: p21, p27 and p57].

Cell cycle

Cell cycle progression is governed by cdks, which are activated by cyclin binding [20,21] and inhibited by the cdk inhibitors [22,23]. The cdks integrate mitogenic and growth inhibitory signals and coordinate cell cycle transitions [24,25]. G₁ to S phase progression is regulated by D-type cyclin-, E-type cyclin- and cyclin A-associated cdks (Fig. 1). B-type cyclin-associated kinases govern G₂ and M phases. Both E-type and D-type cyclin-cdks contribute to phosphorylation of the retinoblastoma protein (pRb). Hypophosphorylated pRb binds members of the E2F and DP1 families of transcription factors, inhibiting these transcriptional activators and actively repressing certain genes. Phosphorylation of pRb in late G₁ phase liberates free E2F/DP1, allowing activation of genes required for S phase (for review [26]).

Cyclin-dependent kinase regulation by phosphorylation

Cdk activation requires phosphorylation of a critical threonine (Thr160 in cdk2 and Thr187 in cdk4). There are two mammalian kinases with *in vitro* cdk activating kinase (CAK) activity: cyclin H/cdk7 and the protein encoded by the human homolog of the *Saccharomyces cerevisiae* CAK1, called Cak1p (for review [27]). The specific roles of these two kinases are somewhat controversial. CAK is active throughout the cell cycle [20,28], but its access to cyclin-bound cdks is inhibited by p27 [29]. Cdks are also negatively regulated by phosphorylation of specific inhibitory sites [27]. Cdc25 phosphatase family members must dephosphorylate these inhibitory sites for full cdk activation. Cdc25A acts on cyclin E-bound cdk2 and is required for G₁ to S phase progression [30].

Cyclin-dependent kinase inhibitors

Two cdk inhibitor families regulate the cell cycle [22,23]. The inhibitor of cdk4 (INK4) family members (p15^{INK4B}, p16^{INK4A}, p18^{INK4C} and p19^{INK4D}) inhibit specifically cdk4 and cdk6. The p16 gene, or MTS1 (Multi-Tumor Suppressor 1), was discovered as a tumour suppressor that is deleted in many cancers [31]. Loss of p15, located near p16 on chromosome 9p, may contribute to loss of G₁ arrest by TGF-β (see below).

The kinase inhibitor protein (KIP) family presently consists of three members, p21^{WAF1/Cip1}, p27^{Kip1} and p57^{Kip2}. The KIPs bind and inhibit a broader spectrum of cdks than do the INK4s. p21 is low in serum-deprived quiescence, but p21 levels and p21 binding to D-type cyclin-cdk complexes increase in early G₁ phase. In addition to regulating G₁ phase progression, p21 acts to coordinate cell cycle responses to DNA damage [23]. p27^{Kip1} was first identified as a heat stable protein whose binding to cyclin E-cdk2 complexes that was increased by TGF-β, lovastatin, or contact inhibition [32,33,34,35,36]. p27 is high in G₀ and early G₁ phase and decreases during G₁ to S phase progression. p27 degradation by ubiquitin-dependent proteolysis [37] is activated by many different growth factors and may involve ras pathways [38–42]. Although cyclin E-cdk2 phosphorylates p27 on Thr187 leading to its degradation in late G₁ phase [43,44], other kinases may also influence p27 function and/or degradation. The possibility that mitogenic signalling pathways that modulate p27 phosphorylation also oppose Smad activation by TGF-β is the subject of intensive investigation.

Although p21 and p27 inhibit cyclin E-cdk2, they also function in the assembly and activation of cyclin D-cdk4 and cyclin D-cdk6 complexes. KIP-mediated assembly of D-type cyclin-cdks in early G₁ phase may facilitate activation of E-type cyclin-cdks through sequestration of KIPs away from cyclin E complexes [45,46].

Mechanisms of cell cycle arrest by TGF-β

TGF-β inhibits phosphorylation of the retinoblastoma protein

Cells are sensitive to TGF-β during a discrete period in early G₁ phase, until they reach a 'restriction point' 6–10 h after G₀ release [47,48]. When TGF-β is added after this critical time point, cells complete the cell cycle but arrest during the subsequent G₁ phase. Laiho *et al* [47] observed that TGF-β inhibits pRb phosphorylation when it is added in early G₁ phase. This key observation suggested that TGF-β was acting before the G₁ to S phase transition to inhibit a pRb kinase, and led to the investigation of TGF-β effects on cell cycle regulators. These studies have shown that TGF-β prevents or inhibits G₁ cyclin-cdk activation through multiple mechanisms, leading to pRb dephosphorylation (Fig. 2). E2F activity is also impaired by TGF-β through a decline in E2F mRNA levels [49]. The observation that E2F overexpression can

prevent TGF- β -mediated arrest [49] emphasizes the importance of the effects of TGF- β on pRb and E2F.

TGF- β downregulates *c-myc*

In many cell types, TGF- β causes a rapid inhibition of *c-myc* transcription [2,16,50]. Transcriptional regulation by the c-Myc protein is required for G₁ to S phase progression. Downregulation of *c-myc* by TGF- β is believed to be important for arrest, because *c-myc* overexpression causes TGF- β resistance [2,51]. Repression of the *c-myc* gene by TGF- β may directly or indirectly contribute to the loss of G₁ cyclins [52,53], to downregulation of Cdc25A [54] and to the induction of the cdk inhibitor p15 [55] (see below).

Effects on G₁/S cyclins

TGF- β causes loss of G₁ cyclins in a cell-type-dependent manner. Cyclin A expression is downregulated by TGF- β in most cell types [32,56] and a TGF- β -regulated region of the cyclin A promoter has been identified [57]. Effects of TGF- β on cyclin E differ among different cell lines. For example, in HaCat keratinocytes TGF- β decreases both mRNA and protein levels of cyclin A and cyclin E, whereas in HMECs cyclin E mRNA is reduced but protein levels are not [32,56]. Although cyclin D₁ levels are decreased by TGF- β in some cell types, this usually occurs late as a consequence of arrest [58,59].

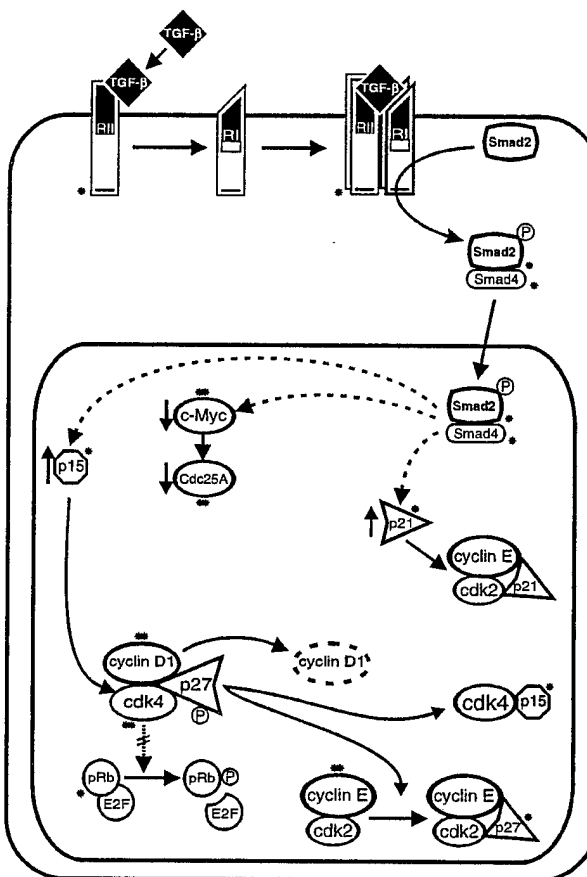
Cooperation between p15 and p27

In epithelial cells, including HMECs, the INK4 and the KIP proteins collaborate to inhibit D-type cyclin-cdks and E-type cyclin-cdks to bring about G₁ arrest by TGF- β [60,61]. *p15^{INK4B}* was first cloned as a gene upregulated by TGF- β [62] and its induction involves an Sp1 site in the promoter [63]. TGF- β induces *p15^{INK4B}* and stabilizes the p15 protein, leading to p15 binding and inhibition of cdk4 and cdk6. Cyclin D₁ and KIP molecules dissociate from cdk4 and cdk6, and p27 accumulates in cyclin E-cdk2 complexes, inhibiting the latter [60,61]. A late downregulation of cyclin D₁ and cdk4 follows G₁ arrest. TGF- β appears to actively regulate p27's affinity for its targets, independent of p15 function, favouring p27 accumulation in cyclin E complexes [61].

Upregulation of p21 expression

In normal HMECs, TGF- β affects neither p21 levels, nor the binding of p21 to target cdks [61]. In other cell types, TGF- β induction of *p21* plays a role in cdk inhibition [59,64,65] and its upregulation is independent of p53 [64,66]. The *p21* gene may be a downstream target of Smad4, because transient overexpression of *Smad4* induces *p21* mRNA [67]. Like *p15*, the *p21^{WAF1/Cip1}* gene promoter contains Sp1 sites that are regulated by TGF- β in reporter gene assays [63,68]. Other cdk inhibitors, p16, p18, p19 and p57, have not to date been implicated in TGF- β -mediated arrest.

Figure 2



Mechanisms of cell cycle arrest by transforming growth factor (TGF)- β and their deregulation in cancer. TGF- β receptor activation leads to Smad2 phosphorylation. Phosphorylated Smad2 then binds Smad4 and the Smad2-Smad4 complex translocates to the nucleus to modulate transcription. Although *p15* and *p21* genes are induced and *c-myc* and *Cdc25A* repressed by TGF- β , these may not be direct effects of Smad2-Smad4 action (dotted lines). TGF- β inhibits G₁ cyclin-cyclin-dependent kinases (cdks) by increasing p15 binding to cdk4 and cdk6 and by increasing p27 (+/- p21) binding to cyclin E-cdk2, thereby inhibiting retinoblastoma protein (pRb) phosphorylation. *Components of the TGF- β effector pathway that are mutated and/or functionally inactivated in human cancers; **molecules whose activation or overexpression may contribute to TGF- β arrest resistance.

Effects on cdk2 phosphorylation

TGF- β also regulates cdk2 phosphorylation. In Mv1Lu cells, TGF- β inhibits cdk2 in part by inhibiting phosphorylation on Thr160 [32,34]. p27 can inhibit CAK access to cyclin-bound cdks *in vitro* [29]. Thus, TGF- β may prevent CAK action by increasing the binding of p27 to cyclin E-cdk2. In HepG2 cells, however, TGF- β inhibits the enzymatic activity of Cak1p [69], indicating an alternative mechanism for the inhibition by TGF- β of Thr160 phosphorylation of cdk2.

Dephosphorylation of inhibitory sites on cyclin E-bound cdk2 is required for G₁ progression and is required for G₁ to S phase progression [30]. In a human breast epithelial line, TGF- β reduced *Cdc25A* expression in association with an increase in inhibitory cdk phosphorylation [54]. The effect on *Cdc25A* expression may be secondary to the repression by TGF- β of *c-myc*, because in some cell types *Cdc25A* is induced by *c-myc* [70].

Loss of TGF- β mediated G₁ arrest in cancer

In nontransformed epithelial cells, TGF- β causes G₁ arrest through downregulation of *c-myc*, inhibition of the G₁ cdks and hypophosphorylation of pRb. Overlapping or redundant cell cycle controls assure growth arrest. In malignant transformed cells, however, this redundancy is often lost and carcinoma-derived cells are usually refractory to growth inhibition by TGF- β [10]. Indeed, in advanced cancers, TGF- β may promote tumour growth and metastatic progression [71]. In this part of the discussion, we review how dysregulation of many different cell cycle mechanisms abrogate TGF- β arrest in cancer (Fig. 2).

Altered cdk inhibitor expression and function

Dysregulation of the INK4 family may contribute to TGF- β resistance in cancer. In human tumours, deletion of *p15* often accompanies *p16* deletion due to their proximity on chromosome 9p [72–74]. Silencing of *p15* through promoter hypermethylation, which is observed in leukaemias, is associated with loss of TGF- β sensitivity [75,76]. In other TGF- β -resistant cells, however, *p15* protein levels may increase normally, indicating that, at least in these lines, a functional *p15* is not sufficient to mediate arrest by TGF- β [65].

Although *p15* and *p27* cooperate to inhibit the G₁ cyclin-cdks in normal cells, neither of these cdk inhibitors are essential for G₁ arrest by TGF- β . *p15* is clearly not essential for TGF- β -mediated G₁ arrest, because cells bearing *p15* deletions can respond through upregulation of *p21* and *p27* [59,65], or downregulation of *Cdc25A* [54]. Lymphocytes from *p27*-null mice can still arrest in response to TGF- β [77]. Nonetheless, the requirement for *p27* in arrest by TGF- β may differ in normal and transformed cells. Although inhibition of *p27* expression through antisense *p27* oligonucleotide transfection did not abrogate TGF- β -mediated arrest in finite lifespan HMECs, it did do so in breast cancer-derived lines (Donovan J, Slingerland J, unpublished data). In normal cells, multiple redundant pathways cooperate to mediate arrest, but in cancer cells the progressive loss of other checkpoints may make *p27* indispensable for TGF- β -mediated arrest.

The antiproliferative role of *p27* is frequently disrupted in human cancers. Although mutations in *p27* are rare [78,79], accelerated proteolysis causes reduced *p27*

protein in many cancers, including breast, and may contribute to TGF- β resistance [37,80–82]. Less often, primary tumours may exhibit strong cytoplasmic *p27* expression associated with poor prognosis. Cytoplasmic *p27* has been observed in some advanced cancer-derived lines [83]. Thus, some cancers may express a stable but inactivated *p27*. In a TGF- β -resistant HMEC line, we have observed stable cytoplasmic *p27* localization, altered *p27* phosphorylation and impaired binding of *p27* to cyclin E-cdk2 (Ciarallo S, Slingerland J, unpublished data). The elucidation of how of mitogenic signalling pathways alter *p27* inhibitor function may prove important insights into mechanisms of TGF- β resistance (see below).

Altered KIP function has also been observed in TGF- β -resistant prostate cancer cells. Although TGF- β caused an upregulation in *p21*-cdk2 binding, this kinase was not inhibited, suggesting that *p21* may not function normally in these cells [84]. Loss of *p21* has also been observed in advanced breast cancers in association with a poor patient prognosis [85,86]. As for *p27*, functional inactivation of *p21* could contribute to TGF- β resistance during breast cancer progression.

Cyclin overexpression and TGF- β resistance

Overexpression and/or amplification of the cyclin D₁ gene is seen in up to 40% of breast cancers [87,88] and could contribute to TGF- β resistance. Indeed, cyclin D₁ transfection of an oesophageal epithelial line conferred resistance to TGF- β [89]. Increased cyclin E protein has also been observed in breast cancers [80,90]. Constitutive overexpression of cyclin E does not confer TGF- β resistance in Mv1Lu cells, however (Slingerland J, Reed S, unpublished data). Pathways that link impaired cyclin degradation with loss of cell cycle responses to TGF- β have yet to be elucidated.

Cdk4 gene amplification and activating mutation

Although loss of *cdk4* does not contribute significantly to arrest by TGF- β because it occurs after most cells have entered G₁ phase [60], ectopic *cdk4* expression can abrogate TGF- β -mediated arrest [91]. The increased *cdk4* level may exceed titration by *p15* and, in addition, sequestration of KIPs away from cyclin E-cdk2 into newly formed cyclin D-cdk4 complexes may lead to loss of *cdk2* inhibition. Overexpression of *cdk4* may contribute to TGF- β resistance in human cancers. Amplification of the *cdk4* gene occurs in primary breast cancers [92] and dominant active *cdk4* mutations have been observed in human malignant melanoma [31].

Activation of *c-myc*, and TGF- β resistance

TGF- β arrest-resistant cells often fail to downregulate *c-myc* [65]. Moreover, oncogenic activation of *c-myc*, which is seen in a number of human malignancies, including breast cancer, may impair TGF- β responsiveness through a number of mechanisms.

Overexpression of *c-Myc* may increase G_1 cyclin levels. *c-Myc* may regulate indirectly the expression of cyclins D_1 , E and A [52,53]. *c-Myc* induction of cyclin D_1 or cyclin D_2 may lead to the sequestration of p27 and p21 away from cyclin E-cdk2, and thus contribute to cyclin E-cdk2 activation [93,94]. These effects, however, which are best demonstrated in fibroblast lines, may not be relevant to TGF- β resistance in epithelial cells. In Mv1Lu cells, *c-myc* overexpression prevents arrest by TGF- β in part by inhibiting *p15* induction [55*]. *c-Myc* effects on D-type cyclin expression and cyclin D-cdk4 complex formation were not sufficient to account for loss of the TGF- β response. Thus, repression of *p15* by *c-Myc* may be important in the arrest-resistant phenotype.

Additional mechanisms link *c-Myc* with cyclin E-cdk2 activation. Overexpression of *c-myc* can induce a heat labile factor that binds p27 and inhibits its association with cyclin E-cdk2 [95]. This effect is independent of p27 degradation. Although in some cell types cyclins D_1 and D_2 may be the *c-myc*-induced inhibitors of p27 [93,94], in other models the *c-myc*-induced inhibitor of p27 appears to be independent of D-type cyclins [95].

Oncogenic activation of *c-myc* may lead to *Cdc25A* overexpression and loss of TGF- β -mediated repression of *Cdc25A* [54*]. Overexpression of *Cdc25A* is observed in primary breast cancers and is associated with a poor patient prognosis (Loda M, personal communication). The increased *Cdc25A* may represent one of the checkpoints whose disruption makes subsequent disruption of p27 function more critical during breast cancer progression.

Activation of Ras and its effector pathways and TGF- β resistance

Overexpression of activated Ras has been shown to abrogate the antimutagenic effects of TGF- β [96]. Mutational activation of *ras* is common in many human cancers and may be linked to TGF- β resistance through a number of mechanisms. Activated Ras can interfere with TGF- β signalling by altering Smad2 phosphorylation and signal transduction [97]. Moreover, Ras activation can increase cyclin D_1 levels through both transcriptional and post-translational mechanisms [38,98,99]. Ras activation also accelerates p27 degradation [40,41], in some models requiring coexpression of Myc [39]. Although *ras* mutations are not commonly observed in breast cancer, epidermal growth factor (EGF) and ErbB2 overexpression are, and both activate the Ras effector phosphatidylinositol-3 kinase [100]. Oncogenic activation of different *ras* effector pathways may abrogate p27 function [41,101], contributing importantly to TGF- β resistance.

Regulation of other G_1 events by TGF- β

p53 may play a role in the TGF- β response in some cells. In murine keratinocytes, introduction of mutated p53 led to

TGF- β resistance, and a correlation between p53 mutation and loss of responsiveness to TGF- β -mediated arrest has been observed in several cancers [102–104].

Constitutive expression of *mdm2* can give rise to TGF- β resistance. Although the Mdm2 protein binds to p53 to mediate p53 proteolysis, the effects of Mdm2 on TGF- β sensitivity appear to be independent of p53 function, because expression of an Mdm2 mutant that failed to bind p53 also conferred resistance [105]. Because overexpression of Mdm2 occurs in about 73% of breast cancers, this too may play a role in TGF- β resistance *in vivo* [85,106,107].

Conclusion

During the past decade the anatomy of cell cycle regulation has been 'worked out'. TGF- β -induced G_1 arrest occurs through induction of *p15* and *p21* genes, repression of the *c-myc*, *Cdc25A*, *cyclin E* and *cyclin A* genes, and an increase in the association of p15, p21 and p27 with target cdks. Inactivation of G_1 cyclin-cdks leads to pRb dephosphorylation and E2F inhibition. The discovery of the Smads as both transducers of TGF- β signalling and transcriptional regulators has been a major advance in this field. Mitogenic signalling via ras/mitogen-activated protein kinase has been shown to interfere with Smad activation [11]. It will be of interest to ascertain whether cross-talk with Smads can also negatively regulate components of mitogenic signal transduction pathways. How growth factors and mitogenic pathways influence the transcriptional activation, intracellular localization and degradation of cyclins and cdk inhibitors is only beginning to be mapped. As these mechanisms are elucidated, we will be able to move from the myopic view of cyclin-cdk regulation in the nucleus, to a broader three-dimensional view of cell cycle regulation that encompasses extracellular and cytoplasmic signalling pathways. The next frontiers lie in the cytoplasm and at the gateway of the nuclear pore as we begin to elucidate how TGF- β /Smad signalling interfaces with transducers of mitogenic signals to regulate cyclin-cdk activities.

Acknowledgements

We thank members of the Slingerland laboratory for critical reading of the manuscript and Ms Sophie Ku for excellent secretarial assistance.

References

Articles of particular interest have been highlighted as:

- of special interest
 - of outstanding interest
1. Massague J, Cheifetz S, Laiho M, et al: Transforming growth factor- β . *Cancer Surv* 1992, 12:81–103.
 2. Alexandrow MG, Moses H: Transforming growth factor β and cell cycle regulation. *Cancer Res* 1995, 55:1452–1457.
 3. Daniel CW, Silberstein GB, van Horn K, Strickland P, Robinson S: TGF- β 1-induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. *Dev Biol* 1989, 135:20–30.

4. Silberstein GB, Daniel CW: Reversible inhibition of mammary gland growth by transforming growth factor- β . *Science* 1987, **237**: 291-293.
 5. Barcellos-Hoff MH, Ewan KB: Transforming growth factor- β and breast cancer: mammary gland development *Breast Cancer Res* 2000, **2**:92-99.
 6. Pierce DFJ, Johnson MD, Matzui Y, et al: Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF- β 1. *Genes Dev* 1993, **7**:2308-2317.
 7. Pierce DF, Gorska AE, Chytil A, et al: Mammary tumor suppression by transforming growth factor β 1 transgene expression. *Proc Natl Acad Sci USA* 1995, **92**:4254-4258.
 8. Wakefield LM: Transforming growth factor- β and breast cancer: lessons learned from genetically altered mouse models. *Breast Cancer Res* 2000, **2**:100-106.
 9. Hosobuchi M, Stampfer M: Effects of the transforming growth factor β on growth of human mammary epithelial cells in culture. *In Vitro Cell Dev Biol* 1989, **25**:705-713.
- A demonstration is provided that normal, finite lifespan HMECs all undergo arrest in response to TGF- β .
10. Fynan TM, Reiss M: Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. *Crit Rev Oncogenesis* 1993, **4**:493-540.
 11. Massague J: TGF- β signal transduction. *Annu Rev Biochem* 1998, **67**:753-791.
 12. Kretschmar M: Transforming growth factor- β and breast cancer: transforming growth factor- β /SMAD signaling defects and cancer. *Breast Cancer Res* 2000, **2**:107-115.
 13. Kerbel RS: Expression of multi-cytokine resistance and multi-growth factor independence in advanced stage metastatic cancer: malignant melanoma as a paradigm. *Am J Pathol* 1992, **141**:519-524.
 14. Massague J: The transforming growth factor- β family. *Ann Rev Cell Biol* 1990, **6**:597-641.
 15. Shipley GD, Pittelkow MR, Wille JJ, Scott RE, Moses HL: Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming growth factor-growth inhibitor in serum-free medium. *Cancer Res* 1986, **46**:2068-2071.
- This study showed that G_1 arrest by TGF- β in normal human prokeratinocytes was reversible.
16. Coffey RJ, Bascom CC, Sipes NJ, et al: Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta. *Mol Cell Biol* 1988, **8**:3088-3093.
 17. Zentella A, Massague J: Transforming growth factor beta induces myoblast differentiation in the presence of mitogens. *Proc Natl Acad Sci USA* 1992, **89**:5176-5180.
- This paper showed that TGF- β mediated irreversible cell cycle arrest and differentiation of myoblasts.
18. Masui T, Wakefield LM, Lechner JF, et al: Type β transforming growth factor is the primary differentiation inducing serum factor for normal human bronchial epithelial cells. *Proc Natl Acad Sci USA* 1986, **83**:2438-2442.
 19. Jetten AM, Shirley JE, Stoner G: Regulation of proliferation and differentiation of respiratory tract epithelial cells by TGF- β . *Exp Cell Res* 1986, **167**:539-549.
 20. Morgan DO: Principles of Cdk regulation. *Nature* 1995, **374**: 131-134.
 21. Sherr CJ: G1 phase progression: cycling on cue. *Cell* 1994, **79**: 551-555.
 22. Sherr CJ, Roberts JM: Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* 1995, **9**:1149-1163.
 23. Sherr CJ, Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999, **13**:1501-1512.
 24. Murray AW: Creative blocks: cell cycle checkpoints and feedback controls. *Nature* 1992, **359**:599-604.
 25. Hartwell L: Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* 1992, **71**:543-546.
 26. Adams PD, Kaelin WG: Transcriptional control by E2F. *Semin Cancer Biol* 1995, **6**:99-108.
 27. Solomon MJ, Kaldis P: Regulation of CDKs by phosphorylation. *Results Probl Cell Diff* 1998, **22**:79-109.
 28. Solomon MJ: Activation of the various cyclin/cdc2 proteins. *Curr Opin Cell Biol* 1993, **5**:180-186.
 29. Kato JY, Matsuoka M, Polyak K, Massague J, Sherr CJ: Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. *Cell* 1994, **79**:487-496.
 30. Draetta G, Eckstein J: Cdc25 protein phosphatases in cell proliferation. *Biochim Biophys Acta* 1997, **1332**:M53-M63.
 31. Bates S, Peters G: Cyclin D1 as a cellular proto-oncogene. *Semin Cancer Biol* 1995, **6**:73-82.
 32. Slingerland JM, Hengst L, Pan C-H, et al: A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor β -arrested epithelial cells. *Mol Cell Biol* 1994, **14**:3683-3694.
- TGF- β caused inhibition of cyclin E-cdk2 and cyclin A-cdk2 through the induction of a heat stable inhibitor that bound to target cdk. This protein was later shown to be p27. TGF- β was also shown to inhibit CAK activation of cdk2 in the Mv1Lu cell line.
33. Hengst L, Dulic V, Slingerland J, Lees E, Reed SI: A cell cycle regulated inhibitor of cyclin dependant kinases. *Proc Natl Acad Sci USA* 1994, **91**:5291-5294.
 34. Koff A, Ohtsuki M, Polyak K, Roberts JM, Massague J: Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF- β . *Science* 1993, **260**:536-539.
- TGF- β inhibits G_1 progression through inhibition of cyclin E-dependent and cyclin A-dependent kinases and through inhibition of the activating phosphorylation of cdk2.
35. Polyak K, Kato JY, Solomon MJ, et al: p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor- β and contact inhibition to cell cycle arrest. *Genes Dev* 1994, **8**:9-22.
- It was demonstrated that TGF- β -mediated arrest involved the action of a 27-kDa protein, p27^{Kip1}, that bound and inhibited cyclin E-cdk2.
36. Polyak C, Lee M-H, Erdjument-Romage H, et al: Cloning of p27KIP1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 1994, **78**:59-66.
 37. Slingerland J, Pagano M: Regulation of the Cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* 2000, **183**:10-17.
 38. Aktas H, Cai H, Cooper GM: Ras links growth factor signalling to the cell cycle machinery via regulation of cyclin D1 and the cdk inhibitor p27^{Kip1}. *Mol Cell Biol* 1997, **17**:3850-3857.
 39. Leone G, DeGregori J, Sears R, Jakoi L, Nevins JR: Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F [published erratum appears in *Nature* 1997, **387**:932]. *Nature* 1997, **387**:422-426.
 40. Kawada M, Yamagoe S, Murakami Y, et al: Induction of p27Kip1 degradation and anchorage independence by Ras through the MAP kinase signaling pathway. *Oncogene* 1997, **15**:629-637.

41. Takuwa N, Takuwa Y: Ras activity late in G1 phase required for p27kip1 downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts. *Mol Cell Biol* 1997, 17:5348-5358.
42. Hu W, Bellone CJ, Baldassare JJ: RhoA stimulates p27 Kip degradation through its regulation of cyclin E/Cdk2 activity. *J Biol Chem* 1999, 274:3396-3401.
43. Vlach J, Hennecke S, Amati B: Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27^{Kip1}. *EMBO J* 1997, 16:5334-5344.
44. Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE: Cyclin E-Cdk2 is a regulator of p27Kip1. *Genes Dev* 1997, 11:1464-1478.
45. LaBaer J, Garret M, Steenson M, et al: New functional activities for the p21 family of cdk inhibitors. *Genes Dev* 1997, 11:847-862.
This study demonstrated a role for KIPs as assembly molecules for cyclin D₁-cdk4 and cyclin D₁-cdk6 complexes. It was shown that KIPs can function to assemble D-type cyclin-cdk complexes *in vitro* and *in vivo*.
46. Cheng M, Olivier P, Diehl JA, et al: The p21(Cip1) and p27(Kip1) CDK inhibitors are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J* 1999, 18:1571-1583.
This paper provides important confirmation of the assembly function of KIP molecules. Mice lacking both p21 and p27 genes were shown to have low levels of cyclin D₁ and to lack cyclin D₁-cdk4 assembly function. Reconstitution of p21 and p27 expression stabilized cyclin D₁ and restored cyclin D-cdk complex formation.
47. Laiho M, DeCaprio JA, Ludlow JW, Livingston DM, Massague J: Growth inhibition by TGF-β1 linked to suppression of retinoblastoma protein phosphorylation. *Cell* 1990, 62:175-185.
This paper was the first to demonstrate that TGF-β inhibits pRb phosphorylation during a limited period in early G₁ phase. It was shown that TGF-β inhibits a pRb kinase and stimulated further investigation of the effects of TGF-β on cyclin-cdk regulation.
48. Howe PH, Draetta G, Leof EB: Transforming growth factor β1 inhibition of p34cdc2 phosphorylation and histone H1 kinase activity is associated with G1/S-phase growth arrest. *Mol Cell Biol* 1991, 11:1185-1194.
49. Schwarz JK, Bassing CH, Kovacs I, et al: Expression of the E2F1 transcription factor overcomes type beta transforming growth factor-mediated growth suppression. *Proc Natl Acad Sci USA* 1995, 92:483-487.
50. Pietenpol JA, Stein RW, Moran E, et al: TGF-β1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* 1990, 61:777-785.
51. Alexandrow MG, Kawabata M, Aakre M, Moses H: Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of transforming growth factor beta-1. *Proc Natl Acad Sci USA* 1995, 92:3239-3243.
This paper demonstrates that c-myc overexpression can abrogate TGF-β-mediated arrest.
52. Jansen-Durr P, Meichle A, Steiner P, et al: Differential modulation of cyclin gene expression by MYC. *Proc Natl Acad Sci USA* 1993, 90:3685-3690.
53. Shibuya HJ, Yoneyama M, Ninomiya-Tsuji J, Matsumoto K, Taniguchi T: IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for c-myc. *Cell* 1992, 70:57-67.
54. Iavarone A, Massague J: Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. *Nature* 1997, 387:417-422.
It was demonstrated that mammary epithelial cells lacking p15 expression can undergo TGF-β-mediated arrest. TGF-β was shown to reduce Cdc25A expression.
55. Warner BJ, Blain SW, Seoane J, Massague J: Myc downregulation by transforming growth factor beta required for activation of the p15(Ink4b) G(1) arrest pathway. *Mol Cell Biol* 1999, 19:5913-5922.
It was shown that TGF-β resistance resulting from Myc overexpression involves the downregulation of p15 expression by c-myc.
56. Geng Y, Weinberg RA: Transforming growth factor β effects on expression of G1 cyclins and cyclin-dependant protein kinases. *Proc Natl Acad Sci USA* 1993, 90:10315-10319.
It was shown that TGF-β causes inhibition of cyclin E-dependent and cyclin A-dependent kinases, in part through inhibition of cyclin E and cyclin A mRNA levels.
57. Feng X-H, Filvaroff EH, Derynck R: Transforming growth factor-beta (TGF-beta)-induced down-regulation of cyclin A expression requires a functional TGF-beta receptor complex. *J Cell Biol Chem* 1995, 270:24237-24245.
58. Ko TC, Sheng HM, Reisman D, Thompson EA, Beauchamp RD: Transforming growth factor-β1 inhibits cyclin D1 expression in intestinal epithelial cells. *Oncogene* 1995, 10:177-184.
59. Florenes VA, Bhattacharya N, Bani MR, et al: TGF-β mediated G1 arrest in a human melanoma cell line lacking p15INK4B: evidence for cooperation between p21Cip1 and p27Kip1. *Oncogene* 1996, 13:2447-2547.
That p15 was not essential for TGF-β-induced cell cycle arrest was demonstrated in a melanoma model.
60. Reynisdottir I, Polyak K, Iavarone A, Massague J: Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. *Genes Dev* 1995, 9:1831-1845.
This study showed that p15 and p27 cooperate to mediate G₁ arrest. Upregulation of a 15-kDa protein in cdk6 complexes was observed. TGF-β caused release of p27 from cdk4 and cdk6 complexes, and an increase in p27 binding to cyclin E-cdk2. In keratinocytes, p21 levels were increased by TGF-β and p21 binding to cdk2 was increased.
61. Sandhu C, Garbe J, Daksis J, et al: Transforming growth factor β stabilizes p15^{INK4B} protein, increases p15^{INK4B}-cdk4 complexes and inhibits cyclin D1/cdk4 association in human mammary epithelial cells. *Mol Cell Biol* 1997, 17:2458-2467.
Cooperation between p15 and p27 was demonstrated in HMECs. TGF-β not only induced p15 expression, but also stabilized p15 protein. Cyclin D₁-cdk4-KIP complexes from a TGF-β-resistant line could not be dissociated by p15 *in vitro*, suggesting that a defect in KIP function may underlie resistance to TGF-β in these cells.
62. Hannon GJ, Beach D: p15^{INK4B} is a potential effector of TGF-β induced cell cycle arrest. *Nature* 1994, 371:257-261.
This paper describes the cloning of the INK4 family member p15 as a gene that is upregulated by TGF-β.
63. Li JM, Nichols MA, Chandrasekharan S, Xiong Y, Wang XF: Transforming growth factor beta activates the promoter of cyclin-dependent kinase inhibitor p15^{INK4B} through an Sp1 consensus site. *J Biol Chem* 1995, 270:26750-26753.
64. Datto MB, Li Y, Panus JF, et al: Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53 independent mechanism. *Proc Natl Acad Sci USA* 1995, 92:5545-5549.
It is demonstrated that p21 is induced by TGF-β in a p53-independent manner.
65. Malliri A, Yeudall WA, Nikolic M, et al: Sensitivity to transforming growth factor β1-induced growth arrest is common in human squamous cell carcinoma cell lines: c-MYC down-regulation and p21^{waf1} induction are important early events. *Cell Growth Differ* 1996, 7:1291-1304.
66. Elbendary A, Berchuck A, Davis P, et al: Transforming growth factor β1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells. *Cell Growth Differ* 1994, 12:1301-1307.
It was demonstrated that p21 is upregulated by TGF-β.

67. Hunt KK, Fleming JB, Abramian A, et al: Overexpression of the tumor suppressor gene Smad4/DPC4 induces p21 waf1 expression and growth inhibition in human carcinoma cells. *Cancer Res* 1998, 58:5656-5661.
68. Datto MB, Hu PP, Kowalik TF, Yingling J, Wang XF: The viral oncoprotein E1A blocks transforming growth factor beta-mediated induction of p21/WAF1/Cip1 and p15/INK4B. *Mol Cell Biol* 1997, 17:2030-2037.
69. Nagahara H, Ezhevsky SA, Vocero-Akbani AM, et al: Transforming growth factor beta targeted inactivation of cyclin E: cyclin-dependent kinase 2 (Cdk2) complexes by inhibition of Cdk2 activating kinase activity. *Proc Natl Acad Sci USA* 1999, 96:14961-14966.
70. Galaktionov K, Chen X, Beach D: Cdc25 cell-cycle phosphatase as a target of c-myc. *Nature* 1996, 382:511-517.
It was shown that c-myc upregulates Cdc25A expression. This effect is not seen in all cell types.
71. Dumont N, Arteaga CL: Transforming growth factor- β and breast cancer: tumor promoting effects of transforming growth factor- β . *Breast Cancer Res* 2000, 2:125-132.
72. Cairns P, Mao L, Merlo A, et al: Rates of p16 (MTS1) mutations in primary tumors with 9p loss. *Science* 1994, 265:415-417.
73. Cairns P, Polascik TJ, Eby Y, et al: Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. *Nature Genet* 1995, 11:210-212.
74. Kamb A, Shattuch-Eidens D, Eetes R, et al: Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nature Genet* 1994, 8:23-26.
75. Batova A, Diccianni MB, Yu JC, et al: Frequent and selective methylation of p15 and deletion of both p15 and p16 in T-cell lymphoblastic leukemia. *Cancer Res* 1997, 57:832-836.
76. Quesnel B, Guillem G, Verecque R, et al: Methylation of the p15 (INK4b) gene in myelodysplastic syndromes is frequent and acquired during disease progression. *Blood* 1998, 91:2985-2990.
77. Nakayama K, Ishida N, Shirane M, et al: Mice lacking p27Kip1 display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 1996, 85:707-720.
78. Kawamata N, Morosetti R, Miller CW, et al: Molecular analysis of the cyclin-dependent kinase inhibitor gene p27Kip1 in human malignancies. *Cancer Res* 1995, 55:2266-2269.
79. Pietenpol JA, Bohlander SK, Sato Y, et al: Assignment of human p27Kip1 gene to 12p13 and its analysis in leukemias. *Cancer Res* 1995, 55:1206-1210.
80. Porter PL, Malone KE, Heagerty PJ, et al: Expression of cell cycle regulators p27kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nature Med* 1997, 3:222-225.
81. Tan P, Cady B, Wanner M, et al: The cell cycle inhibitor p27 is an independent prognostic marker in small (T1a,b) invasive breast carcinomas. *Cancer Res* 1997, 57:1259-1263.
82. Catzavelos C, Bhattacharya N, Ung YC, et al: Decreased levels of the cell cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. *Nature Medicine* 1997, 3:227-230.
83. Orend G, Hunter T, Ruoslahti E: Cytoplasmic displacement of cyclin E-cdk2 inhibitors p21Cip1 and p27Kip1 in anchorage-independent cells. *Oncogene* 1998, 16:2575-2583.
84. Cipriano SC, Chen YQ: Insensitivity to growth inhibition by TGF-beta1 correlates with a lack of inhibition of the CDK2 activity in prostate carcinoma cells. *Oncogene* 1998, 17:1949-1556.
85. Jiang M, Shao Z-M, Wu J, et al: p21/waf1/cip1 and mdm-2 expression in breast carcinoma patients as related to prognosis. *Int J Cancer* 1997, 74:529-534.
86. Tshilias J, Kapusta LR, DeBoer G, et al: Loss of cyclin dependent kinase inhibitor p27Kip1 is a novel prognostic factor in localized human prostate adenocarcinoma. *Cancer Res* 1998, 58:542-548.
87. Lammie GA, Fantl V, Smith R, et al: D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. *Oncogene* 1991, 6:439-444.
88. Buckley MF, Sweeney KJE, Hamilton JA, et al: Expression and amplification of cyclin genes in human breast cancer. *Oncogene* 1993, 8:2127-2133.
89. Okamoto A, Jiang W, Kim SJ, et al: Overexpression of human cyclin D1 reduces the transforming growth factor beta (TGF-beta) type II receptor and growth inhibition by TGF-beta 1 in an immortalized human esophageal epithelial cell line. *Proc Natl Acad Sci USA* 1994, 91:11576-11580.
90. Nielsen N, Amerlov C, Emdin S, Landberg G: Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to estrogen receptor status. *Br J Cancer* 1996, 74:874-880.
91. Ewen ME, Sluss HK, Whitehouse LL, Livingston DM: TGF- β inhibition of cdk4 synthesis is linked to cell cycle arrest. *Cell* 1993, 74:1009-1020.
Overexpression of cdk4, but not of cdk2, was shown to abrogate sensitivity to G₁ arrest by TGF- β .
92. An H-X, Beckmann MW, Reifemberger G, Bender HG, Niederacher D: Gene amplification and overexpression of Cdk4 in sporadic breast carcinomas is associated with high tumor cell proliferation. *Am J Pathol* 1999, 154:113-118.
93. Perez-Roger I, Kim SH, Griffiths B, Sweing A, Land H: Cyclins D1 and D2 mediate Myc-induced proliferation via sequestration of p27Kip1 and p21 Cip1. *EMBO J* 1999, 18:5310-5320.
94. Bouchard C, Thisted K, Maier A, et al: Direct induction of cyclin D2 by Myc contributes to cell cycle induction and sequestration of p27. *EMBO J* 1999, 18:5321-5333.
95. Vlach J, Hennecke S, Alevizopoulos K, Conti D, Amati B: Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. *EMBO J* 1996, 15:6595-6604.
96. Filmus J, Zhao J, Buick RN: Overexpression of H-ras oncogene induces resistance to the growth-inhibitory action of transforming growth factor beta-1 (TGF-beta 1) and alters the number and type of TGF-beta 1 receptors in rat intestinal epithelial cell clones. *Oncogene* 1992, 7:521-526.
97. Kretschmar M, Doody J, Timokhina I, Massague J: A mechanism of repression of TGF-beta/Smad signaling by oncogenic Ras. *Genes Dev* 1999, 13:804-816.
98. Cheng M, Sexl V, Sherr CJ, Roussel MF: Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). *Proc Natl Acad Sci USA* 1998, 95:1091-1096.
99. Diehl JA, Cheng M, Roussel MF, Sherr CJ: Glycogen synthase kinase-3 beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998, 12:3499-3511.
100. Ram TG, Ethier SP: Phosphatidylinositol 3-kinase recruitment by p185erbB-2 and erbB-3 is potentially induced by neu differentiation factor/hergulin during mitogenesis and is constitutively elevated in growth factor-independent breast carcinoma cells with c-erbB-2 gene amplification. *Cell Growth Differ* 1996, 7:551-561.

101. Brennan P, Babbage JW, Burgering BMT, *et al*: Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity* 1997, 7:679-689.
102. Gerwin BI, Spillare E, Forrester K, *et al*: Mutant p53 can induce tumorigenic conversion of human bronchial epithelial cells and reduce responsiveness to a negative growth factor, transforming growth factor β 1. *Proc Natl Acad Sci USA* 1992, 89:2759-2763.
103. Reiss M, Vellucci VF, Zhou ZL: Mutant p53 tumor suppressor gene causes resistance to transforming growth factor beta 1 in murine keratinocytes. *Cancer Res* 1993, 53:899-904.
104. Wyllie FS, Dawson T, Bond JA, *et al*: Correlated abnormalities of transforming growth factor- β 1 response and p53 expression in thyroid epithelial cell transformation. *Mol Cell Endocrinol* 1991, 76:13-21.
105. Sun P, Dong P, Dai K, Hannon GJ, Beach D: p53-independent role of MDM2 in TGF- β 1 resistance. *Science* 1998, 282:2270-2272.
106. Bueso-Ramos CE, Manshouri T, Haidar MA, *et al*: Abnormal expression of MDM-2 in breast carcinomas. *Breast Cancer Res Treat* 1996, 37:179-188.
107. Gunther T, Schneider-Stock R, Rys J, Niezabitowski A, Roessner A: p53 gene mutations and expression of p53 and mdm2 proteins in invasive breast carcinoma. A comparative analysis with clinicopathological factors. *J Cancer Res Clin Oncol* 1997, 123:388-394.

Authors' affiliations: Jeffrey Donovan (Department of Medical Biophysics, University of Toronto, and Division of Cancer Research, Toronto Sunnybrook Regional Cancer Centre and Sunnybrook and Women's College Health Sciences Centre, Ontario, Toronto, Canada) and Joyce Slingerland (Departments of Medicine and Medical Biophysics, University of Toronto, and Division of Cancer Research, Toronto Sunnybrook Regional Cancer Centre and Sunnybrook and Women's College Health Sciences Centre, Ontario, Toronto, Canada)

Correspondence: Joyce Slingerland, Division of Cancer Research, Sunnybrook and Women's College Health Sciences Centre, 2075 Bayview Ave, Toronto, Ontario, Canada M3N 3M5.
Tel: +416 480 6100, ext 3494; fax: +416 480 5703;
e-mail: joyce.slingerland@utoronto.ca

Down-regulation of p21^{WAF1/CIP1} or p27^{Kip1} abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells

Sandrine Cariou^{*†}, Jeffrey C. H. Donovan^{*†}, W. M. Flanagan^{*§}, Andrea Milic[†], Nandita Bhattacharya[†], and Joyce M. Slingerland^{*†¶}

^{*}Division of Cancer Biology Research, Sunnybrook and Women's College Health Science Centre, 2075 Bayview Avenue, Toronto, ON, Canada M4N 3M5; and [†]Gilead Sciences, 346 Lakeside Drive, Foster City, CA, 94404

Edited by Joan V. Ruderman, Harvard Medical School, Boston, MA, and approved May 31, 2000 (received for review January 14, 2000)

Estrogens and antiestrogens influence the G₁ phase of the cell cycle. In MCF-7 breast cancer cells, estrogen stimulated cell cycle progression through loss of the kinase inhibitor proteins (KIPs) p27 and p21 and through G₁ cyclin-dependent kinase (cdk) activation. Treatment with antiestrogen drugs, Tamoxifen or ICI 182780, caused cell cycle arrest, with up-regulation of both p21 and p27 levels, an increase in their binding to cyclin E-cdk2, and kinase inhibition. The requirement for these KIPs in the arrests induced by estradiol depletion or by antiestrogens was investigated with antisense. Antisense inhibition of p21 or p27 expression in estradiol-depleted or antiestrogen-arrested MCF-7 led to abrogation of cell cycle arrest, with loss of cyclin E-associated KIPs, activation of cyclin E-cdk2, and S phase entrance. These data demonstrate that depletion of either p21 or p27 can mimic estrogen-stimulated cell cycle activation and indicate that both of these KIPs are critical mediators of the therapeutic effects of antiestrogens in breast cancer.

Estrogen is mitogenic in up to 50% of *de novo* breast cancers, causing recruitment of quiescent cells into G₁ and shortening the G₁-to-S phase interval (1, 2). Although 70% of breast cancers express the estrogen receptor (ER), only two-thirds of these will respond to antiestrogens, of which, Tamoxifen is the most widely used (3, 4). Antiestrogens, such as Tamoxifen, its active metabolite, 4-hydroxytamoxifen (4-OH TAM), and the more potent steroidal antiestrogen ICI 182780 (Faslodex) lead to a G₀/G₁ arrest in susceptible ER-positive breast cancer cells (5–8). Unfortunately, hormonally responsive breast cancers invariably develop resistance to antiestrogens despite the continued expression of wild-type ER in most cases (9–12). Estrogens induce conformational changes in the ER, which promote its nuclear localization, dimerization, and function as a ligand-activated transcription factor (13–15). In addition, ligand binding to the ER can rapidly and transiently activate signal transduction pathways, notably the mitogen-activated protein kinase in breast cancer and in other cell types (16, 17). Because antiestrogen resistance usually develops in the presence of an intact ER, the mechanisms by which ER modulates the cell cycle may be altered during breast cancer progression. The evolution of prostate cancer to hormone independence also occurs without loss of the androgen receptor (18, 19) and may reflect a common mechanism of cell cycle misregulation.

Progression through the cell cycle is governed by a family of cyclin-dependent kinases (cdks), whose activity is regulated by phosphorylation (20), activated by cyclin binding (21, 22), and inhibited by the cdk inhibitors of the inhibitor of cdk4 (INK4) family (p16^{INK4A}, p15^{INK4B}, p18, and p19) and kinase inhibitor protein (KIP) family (p21^{WAF1/CIP1}, p27^{Kip1}, and p57^{KIP2}; refs. 22–24). Passage through G₁ into S phase is regulated by the activities of cyclin D-, cyclin E-, and cyclin A-associated kinases. Although p27 protein is strongly expressed in normal mammary epithelial tissue, decreased levels of p27 protein in primary breast cancers are correlated with poor prognosis (25, 26) and steroid independence (25). Reduced p21 levels have also been associated with a poor prognosis in some breast cancer studies

(27–29). Expression of the ER, a good prognostic factor in breast cancer, is associated with higher levels of both p21 and p27 proteins (25, 27, 28, 30). Our observation that loss of p27 was strongly associated with hormone independence (25) stimulated the present investigation of the role of these KIPs in cell cycle effects of estrogen and antiestrogens in breast cancer cells.

Although recent reports correlate estrogenic stimulation with activation of cyclin E-cdk2, some suggest the importance of the cdk inhibitor p21 (31, 32) and others emphasize a role for p27 (33). An understanding of how estrogens and antiestrogens influence the cell cycle and the mechanisms of their alteration in cancer progression may facilitate the development of new hormonal treatments for breast cancer and other hormone-dependent cancers. The present study provides evidence that both p21 and p27 play essential roles in the cell cycle arrest of breast cancer cells by antiestrogens.

Materials and Methods

Cell Culture and Synchronization. MCF-7 cells (34) were grown in improved modified essential medium (IMEM-option Zn²⁺) supplemented with insulin and 5% (vol/vol) FCS. Cells were transferred to phenol red-free medium for 48 h and then synchronized in quiescence by depletion of estradiol through transfer to IMEM-option Zn²⁺ supplemented with 5% (vol/vol) charcoal-stripped FCS for 48 h.

Analysis of Cell Cycle Regulators. Cells were released from quiescence by readdition of 10 nM 17- β -estradiol (estradiol) at $t = 0$ h. At intervals thereafter, cells were harvested for cell cycle analysis by flow cytometry after BrdUrd pulse labeling and propidium iodide counterstaining (35) and for Western analysis of cyclins E, D1, A, and B, as well as cdk2, cdk4, cdk6, p15, p21, and p27 proteins (from 20–50 μ g of protein extract) as described (36). Equal protein loading was verified by probing these blots with antibody to β -actin (Sigma). Quantitation of proteins on Western and immunoprecipitation–Western blots was done by densitometry with IMAGEQUANT software. Cyclin E was immunoprecipitated from 100–150 μ g of protein lysate with anti-cyclin E mAb 172 and associated proteins detected by immunoblotting or associated kinase assayed with histone H1 as substrate as described (36). At the times indicated,

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: cdk, cyclin-dependent kinase; KIP, kinase inhibitor protein; 4-OH TAM, 4-hydroxytamoxifen; INK4, inhibitor of cdk4; ER, estrogen receptor.

^{*}S.C. and J.C.H.D. contributed equally to this work.

[§]Present address: Sunesis Pharmaceuticals, Redwood City, CA 94063.

[¶]To whom reprint requests should be addressed. E-mail: joyce.slingerland@utoronto.ca.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.160016897. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.160016897

Cdk4 immunoprecipitates (polyclonal antibody from Santa Cruz Biotechnology) were analyzed for associated proteins, and cyclin D1 was immunoprecipitated from 200 μ g of protein with DCS-11 antibody (Neomarkers, Fremont, CA) for kinase assays with pRb substrate as described (37). Immunohistochemical analysis of p27 was undertaken at the indicated times after addition of estradiol as described (25).

Analysis of Cell Cycle Arrest by Inhibition of ER Signaling. Asynchronous MCF-7 cultures were either depleted of estradiol as described above or arrested by addition of 1 μ M 4-OH-TAM (Sigma) or 10 nM ICI 182780 {7 α -(4,4,5,5,5-pentafluoropentylsulfanyl)nonyl]estra-1,3,5(10),-triene-3,17 β -diol; Zeneca Pharmaceuticals, Wilmington, DE} to complete medium, and samples were collected at 0, 12, 18, 24, and 48 h thereafter for protein and flow cytometric analyses. p21, p27, cyclins E, and D1 proteins were assayed by Western analysis, and cyclin E-associated cdk2, p21, p27, and histone H1 kinase activities were assayed as in ref. 36.

Antisense Oligonucleotide Transfections. Phosphorothioate oligonucleotide sequences were as follows: GS5422 antisense p27 (ASp27), 5'-TGGCTCTCTGCGCC-3'; GS5585 mismatch p27 (MSMp27), 5'-TGGCTCXCTTGC GCC-3'; GS6008 antisense p21 (ASp21), 5'-TCCGXGCCAGCTCC-3'; GS6074 mismatch p21 (MSMp21), 5'-TCCGXCGCCAGCTCC-3'. X indicates the G clamp modification of these oligonucleotides (38). MCF-7 cells rendered quiescent by estradiol depletion or by treatment with 10 nM ICI 182780 or 1 μ M 4-OH TAM were transfected with 120 nM oligonucleotides by using 2.5 μ g/ml cytofectin G3815 (Gilead Scientific, Foster City, CA) for 6 h as described (38, 39), followed by replacement with complete medium. Flow cytometry and protein analysis were performed at 1, 21, and 28 h thereafter. Neither p21 nor p27 was increased in mismatch controls compared with lipid-only transfections.

Metabolic Labeling. Before and at 1 h and 21 h after completion of antisense oligonucleotide transfection, cells were metabolically labeled, followed by immunoprecipitation of p21 and p27. Cells were incubated for 30 min in 8 ml of α -MEM lacking methionine and then labeled metabolically for 1 h with 500 μ Ci [35 S]methionine in 2 ml of α -MEM lacking methionine. Cells were lysed on ice, clarified by centrifugation, and precleared with 1 μ g of nonspecific rabbit polyclonal IgG and protein A Sepharose beads. [35 S]Methionine incorporation was quantitated as trichloroacetic acid-insoluble counts. Lysates volumes representing equal amounts (10^8 cpm) of total trichloroacetic acid-incorporated counts were immunoprecipitated for 1 h with either p21 or p27 antibody or with control nonspecific rabbit polyclonal IgG. Immune complexes were collected on protein A Sepharose beads, washed three times, and eluted into Laemmli buffer. Samples were resolved by SDS/PAGE; gels were dried; and labeled proteins were visualized by autoradiography.

Results and Discussion

Estradiol stimulates a shift of KIP proteins from cyclin E-cdk2 into cyclin D1-cdk4. Estradiol stimulation of steroid-deprived, quiescent MCF-7 breast cancer cells induced synchronous cell cycle reentry. S phase entrance was detected by 12 h, with the peak percentage of S phase cells at 24 h (Fig. 1A). The levels of cyclin E, cdk2, and cdk4 remained constant, and p15^{INK4B} protein levels fell as cells moved into G₁ (data not shown). MCF-7 cells do not express p16^{INK4A} because of deletion of this gene (40). Cyclin D1 was not detected in quiescent cells but rose within 3 h of estradiol addition and remained constant thereafter. p21 and p27 protein levels fell by 3- and 5-fold, respectively, by 24 h (Fig. 1B). Immunohistochemical analysis of p27 supported the immunoblotting data (Fig. 1C). Estradiol-depleted, quiescent MCF-7 cells showed strong nuclear

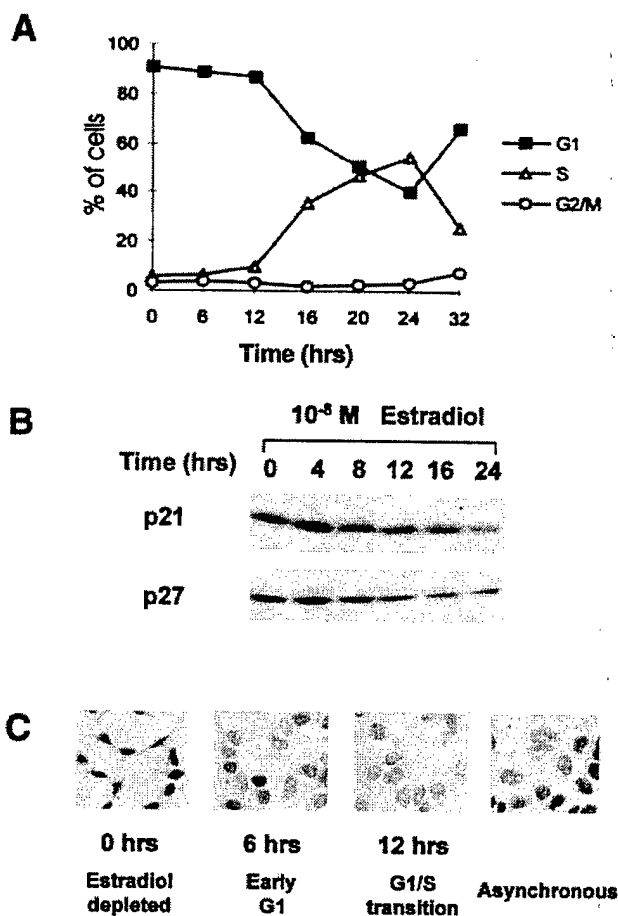


Fig. 1. Losses of p21 and p27 during estradiol stimulation of quiescent MCF-7 cells. Quiescent, estradiol-depleted MCF-7 cells were stimulated by readdition of 10 nM estradiol, and samples were taken at intervals thereafter. (A) Cell cycle synchrony was determined by dual BrdUrd/propidium iodide pulse labeling and flow cytometric analysis. (B) p21 and p27 immunoblots revealed levels of these proteins during cell cycle progression. (C) p27 protein was assayed by immunohistochemistry in asynchronous cultures at the indicated times after estradiol stimulation of quiescent MCF-7 as described (25).

p27 staining that was notably reduced by 6 h after addition of estradiol and barely detectable above background by 12 h as S phase entrance began.

The pattern of binding of p21 and p27 to cdk4 and cdk2 complexes differed during estradiol-stimulated cell cycle progression in MCF-7. Cdk4-bound p27 was abundant in estradiol-depleted cells and increased in parallel with the increased assembly and activation of cyclin D1-cdk4 between 3 and 9 h after estradiol addition (Fig. 2A and B). Cyclin D1-dependent kinase activities and cyclin D1 binding to cdk4 were reduced by 12 h and undetectable by 16 h. Although p21 protein was elevated, very little p21 was detectable in cdk4 complexes in quiescent MCF-7. As for p27, cyclin D1-cdk4 assembly was accompanied by increased p21 binding, in keeping with the function of p21 and p27 as positive regulators of cyclin D1-cdk4 assembly (37, 41). Although cyclin D2 and cdk6 are detectable in MCF-7, cyclin D1-cdk4 has been shown to be the major D-type cdk in these cells (31, 32). In contrast to their pattern in cyclin D1 complexes, activation of cyclin E-cdk2 after estradiol stimulation was correlated with loss of p27 and p21 from cyclin E-cdk2. Cyclin E-cdk2 activation was correlated with S phase entrance (Fig. 2B and C).

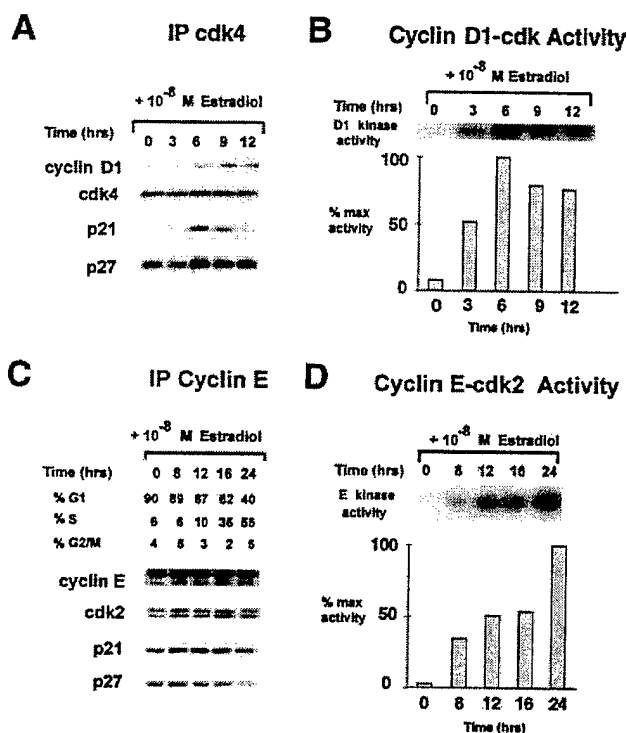


Fig. 2. Different patterns of KIP binding during cyclin D1-cdk4 and cyclin E-cdk2 activation. Cdk4 (A) and cyclin E (C) immunoprecipitates (IP) from cell lysates recovered at intervals after readdition of estradiol to steroid-depleted MCF-7 cells were resolved and analyzed by immunoblotting with the indicated antibodies. Cyclin D1 (B) and cyclin E (D) immunoprecipitates were assayed for kinase activity (36) at intervals after estradiol stimulation of quiescent MCF-7.

p21 and p27 Bind and Inhibit Cyclin E-cdk2 on Interruption of Mitogenic ER Signaling. To investigate further cell cycle regulation by estrogen, ER signaling was interrupted in asynchronous MCF-7 cultures in three ways: by treatment with the pure ER antagonist ICI 182780, by the addition of antiestrogen 4-OH TAM, or by steroid depletion. All induced quiescence, with the S phase fraction falling from 29–36% to 1–5% over 48 h with a corresponding increase in the percentage of cells in G₀/G₁ phase (data shown for ICI 182780 in Fig. 3). p21 and p27 proteins increased, as did their binding to cyclin E-cdk2, in parallel with kinase inhibition (Fig. 3B). Although levels of cyclin E-bound cdk2 were unchanged, there was an accumulation of the slower-mobility, non-CAK-activated cdk2, lacking Thr-160 phosphorylation. The pattern of increase in p21 and p27 and of their binding to cyclin E during estradiol depletion and 4-OH TAM arrest were similar to that shown for ICI 182780 in Fig. 3B. The loss of cyclin D1 observed during antiestrogen treatment would lead to dissociation of KIPs from cyclin D1 complexes and foster KIP inhibition of cyclin E-cdk2 (42, 43).

These data and earlier work support the notion that estrogens and antiestrogens work through changes in p21 and p27 levels. Foster and Wimalasen (33) showed that p27 immunoprecipitation significantly depleted cyclin E-cdk2 inhibitory activity from serum and amino acid-starved MCF-7 cells. Others showed that most of the cyclin E-cdk2 inhibitory activity in Tamoxifen- or ICI 182780-arrested MCF-7 was removed by immunodepletion of p21. However, immunodepletion of both p21 and p27 was required to deplete fully cyclin E from arrested cells, indicating that cyclin E is bound to either p21 or p27 in an ER-blocked arrest state (31, 32, 42). These authors proposed that the estradiol-stimulated up-regulation of cyclin D1 served to seques-

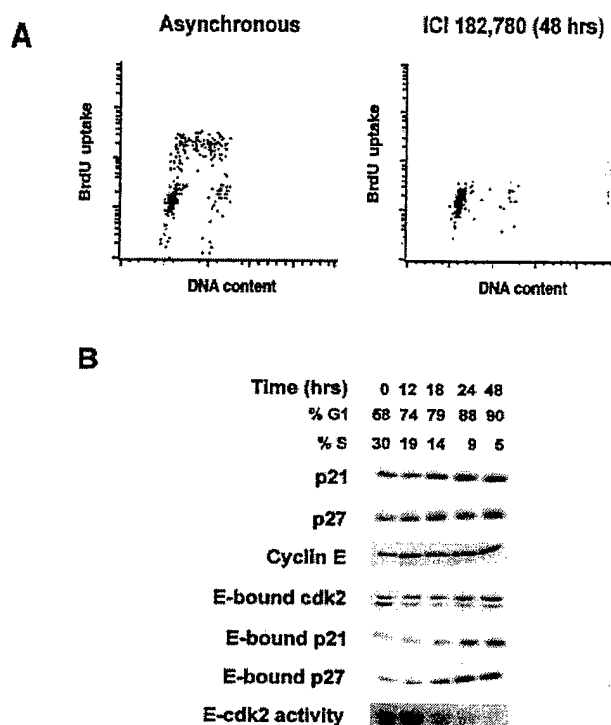


Fig. 3. p21 and p27 proteins increase during G₀/G₁ arrest by ER blockade. Asynchronously growing MCF-7 cells were treated with the ER-blocking drug ICI 182780 (Faslodex) at time 0 h, and samples were collected for flow cytometry or protein analysis at times indicated. (A) Cell cycle distribution before and 48 h after drug treatment. (B) Lysates were analyzed by immunoblotting with the indicated antibodies. Cyclin E immunoprecipitates were immunoblotted for associated p21 or p27 or analyzed for associated histone H1 kinase activity as described in ref. 36. Similar results were obtained for arrests with 4-OH TAM or after transfer to estradiol-depleted, charcoal-stripped serum.

ter p21 away from cyclin E complexes leading to activation of cyclin E and pRb phosphorylation. However, none of these earlier studies definitively established a requirement for p21 and p27 in cell cycle arrest by antiestrogens.

p21 and p27 Are Essential Mediators of Arrest by Antiestrogens. To test directly whether p21 and p27 play essential roles in the cell cycle arrest after blockade of ER signaling in MCF-7 cells, we used antisense oligonucleotides to inhibit p21 (ASp21) or p27 (ASp27) expression in cells arrested by ICI 182780, 4-OH TAM, or estradiol depletion (data shown for ASp27 in estradiol-depleted MCF-7 in Fig. 4). A G clamp heterocycle modification, a cytosine analog that clamps onto a guanine, was designed to enhance antisense/RNA interaction and showed increased antisense oligonucleotide potency over the C5 propynyl-modified oligonucleotides used in earlier assays (38, 39). Within 1 h of transfection, ASp27-treated cells showed a 4-fold reduction in p27 but no loss of p21 (Fig. 4A), and p27 levels reached a nadir at about 6 h after transfection. The ASp21 oligos showed a similar specificity with no immediate loss of p27. Metabolic pulse labeling of ASp27-transfected cells showed specific inhibition of p27 synthesis but no effect on p21 protein synthesis at 1 and 21 h after completion of the transfection (Fig. 4A).

Treatment with ASp27 led to hyperphosphorylation of pRb and p130, CAK phosphorylation at Thr-160 on cdk2 (Fig. 4B), loss of p27 binding to cyclin E-cdk2, and cyclin E-cdk2 activation (Fig. 4C and D), all consistent with stimulation of G₁-to-S phase progression. Similar findings were observed for ASp21-

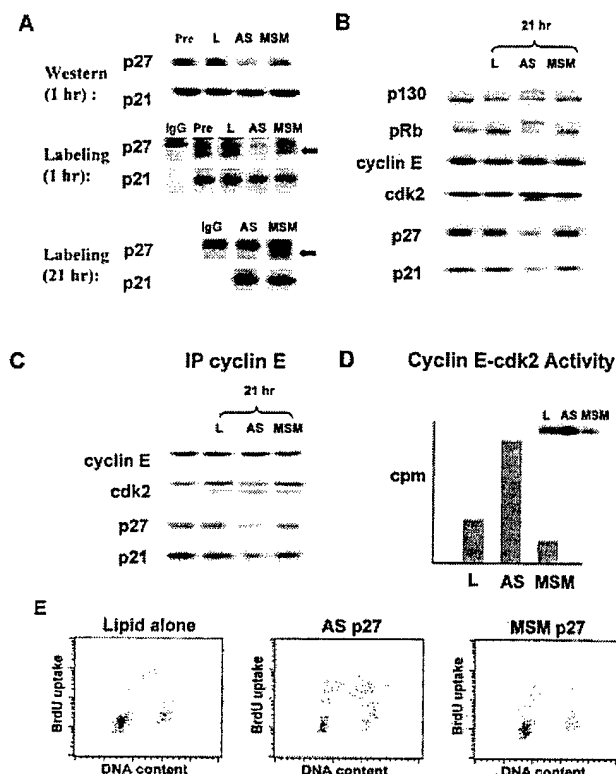


Fig. 4. Requirement of p27 for cell cycle arrest by estradiol depletion. (A) Estradiol-depleted MCF-7 cells were lysed before (left lane) or 1 h after exposure to lipid only (L), ASp27 (AS), or MSMp27 (MSM) and were immunoblotted for p21 and p27 at 1 and 21 h after ASp27 transfection, cells were metabolically pulse labeled with [35 S]methionine, and p21 and p27 were immunoprecipitated from lysates containing equal trichloroacetic acid incorporation. The positions of metabolically labeled p27 are indicated by arrows. A nonspecific band, migrating close to p27 in all lanes, including the control nonspecific IgG lane. (B) Immunoblotting shows cell cycle regulatory protein levels before (left lane) or 21 h after transfection of lipid alone (L), ASp27, or MSMp27. Cyclin E immunoprecipitates (IP) were recovered from the same lysates as in B above and immunoblotted to detect associated proteins (C) or assayed for cyclin E-associated histone H1 kinase activity (D). (E) Flow cytometry 21 h after transfection with ASp27, lipid alone, or MSMp27.

treated cells. ASp21 treatment of cells arrested by estradiol-depletion, 4-OH TAM, or ICI 182780 showed loss of p21 and loss of p21 from cyclin E-cdk2 with activation of this kinase accompanying S phase entry (not shown). These effects were not observed in the mismatch and lipid control groups. Results shown are representative of up to three repeat experiments. It is notable that ASp27-treated cells showed late down-regulation of p21 at 21 h after transfection. Metabolic labeling of ASp27-transfected cells showed persistent specific inhibition of p27 synthesis at 21 h but no inhibition of p21 synthesis as cells were entering S phase. Thus, the reduction of p21 protein is not due to inhibition of p21 synthesis by the ASp27 oligo. Rather, the ASp27-induced inhibition of p27 synthesis was sufficient to move cells out of quiescence, and the subsequent loss of p21 most likely reflects the changes in posttranslational regulation of p21 leading to its degradation at the G₁-to-S phase transition (44). Data in Fig. 4 A–C for ASp27 treatment of ICI 182780-arrested cells were similar to results for ASp27 treatment of cells arrested by estradiol depletion or 4-OH TAM (not shown). Despite the continued blockade of ER signaling, ASp21 or ASp27 transfection stimulated cell cycle entry of cells arrested by steroid depletion, 4-OH TAM, or ICI 182780. The S phase fraction rose

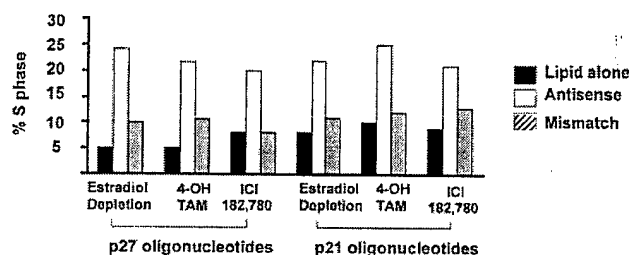


Fig. 5. Requirement for p21 and p27 in G₁ arrest by ER-blocking drugs or estradiol-depletion. MCF-7 cells were arrested by estradiol depletion or by treatment with 4-OH TAM or ICI 182780. The graph indicates the percentage of S phase cells after a 21-h exposure to lipid only (black bars), antisense (white bars), or mismatch (hatched bars) oligonucleotides to either p21 or p27.

to 21–26% at 21 h (or 15 h after reaching minimum levels of the AS-targeted KIP protein, Figs. 4E and 5) and 29–36% at 28 h after transfection. Thus, ASp21 or ASp27 was sufficient to mimic the effect of estradiol on G₁-to-S phase progression in MCF-7 cells. These data indicate that a key effect of ER signaling is to relieve KIP-mediated inhibition of cdk2.

ASp27 not only inhibits p27 synthesis but would also lead to an increase in p27 degradation. As cyclin E-cdk2 is liberated from bound p27, it then phosphorylates remaining p27 molecules on Thr-187, thereby accelerating p27 degradation (45–47). Moreover, the activation of cyclin E-cdk2 is autocatalytic through activation of Cdc25A by cyclin E-cdk2 (48, 49). Thus, a relatively small initial reduction in p27 can stimulate a significant loss of p27 from cyclin E complexes.

Although breast cancer cells arrested by interruption of ER signaling have high levels of both p21 and p27, only p27 is elevated in serum-starved fibroblasts, and p27 is essential for arrest by serum starvation in immortalized fibroblasts (50, 51). Although p21 can compensate in part for the lack of p27 in serum-starved p27-null murine embryo fibroblasts (52), the mechanisms of quiescence induced by serum starvation in fibroblasts and those induced by steroid depletion in malignant breast epithelial cells differ importantly. Our data demonstrate that the cell cycle arrest induced by estradiol depletion or ER blockade requires both p21 and p27 these KIPs are not merely up-regulated as a consequence of cell cycle arrest.

Mitogenic effects of estradiol include the up-regulation of cyclin D1 through increased transcription (42, 43) and stabilization of cyclin D1 protein by assembly with cdk4 and cdk6 (53, 54), the latter mediated by p21 and p27 (37, 41). In addition, the present data establish definitively that estradiol-mediated losses of p21 and p27 relieve the inhibition of cyclin E-cdk2 (31, 32, 42).

In estradiol-depleted MCF-7, although p21 and p27 were abundant, they were not competent to stabilize cyclin D1 via assembly with its cdk partners. Cyclin D1 synthesis is detectable in metabolically labeled quiescent breast epithelial cells, but its association with cdk4 or cdk6 is detectable only several hours after mitogenic stimulation (ref. 36 and S. Cariou and J. Slingerland, unpublished results). Thus, an important effect of estradiol may be the conversion of p21 and p27 from a form that does not support assembly of cyclin D-cdk complexes in G₀, to one that does. Similarly, in serum-starved fibroblasts, p27 did not support cyclin D1-cdk4 complex formation even after ectopic cyclin D1 overexpression (55). Moreover, in p21/p27 null cells, overexpression of cyclin D1 did not permit its assembly with cdk4 (41). After estradiol stimulation in MCF-7, KIP-cyclin D1-cdk4 assembly occurred at 6–9 h, whereas the loss of p27 and p21 from cyclin E complexes was notable only somewhat later, after the time of peak sequestration of these KIPs in cyclin D1 complexes. Although induced overexpression of cyclin D1 can abrogate

antiestrogen arrest (42, 43), the physiologic up-regulation of cyclin D1 stimulated by estradiol in MCF-7 may be insufficient, on its own, to mediate the shifts of the KIPs out of cdk2.

The ubiquitin-mediated degradation of p27 (56–59) requires its phosphorylation on Thr-187 (45–47). Degradation of p21 is also proteasome dependent but may differ importantly from that of p27 (44). Although cyclin E-cdk2 acts *in vitro* and *in vivo* to phosphorylate p27 on Thr-187, other kinases may act on p27 before its degradation. The transition of p21 and p27 from potent inhibitors of cyclin E-cdk2 in G₀ to cyclin D-dependent kinase assembly factors may require phosphorylation early in G₁, before cyclin E-cdk2 activation. We have observed an increase in p27 phosphorylation before the reduction in its steady-state levels in estradiol-stimulated MCF-7 (S. Cariou and J. Slingerland, unpublished results). Mitogen-activated protein kinase activation has been implicated in p27 degradation (60, 61). It will be of interest to determine whether the estradiol-dependent activation of mitogen-activated protein kinase (16, 17) or other mitogenic kinase pathways regulate the transition of p21 and p27 from

high-affinity inhibitors of cyclin E-cdk2, to activators of cyclin D1-cdk assembly.

The approximation that over 4 million women with breast cancer are on Tamoxifen worldwide is a minimal estimate (refs. 3 and 4 and V. C. Jordan, personal communication). An increasing body of *in vitro* data and metaanalysis of large patient cohorts have confirmed the requirement for ER expression for the therapeutic efficacy of Tamoxifen (3, 4). The present study suggests that, in addition to the ER, a breast cancer cell must express functional p21 and p27 for Tamoxifen or Faslodex (ICI 182780) to mediate cytostatic effects. These observations raise the hypothesis that deregulation and loss of these KIPs may underlie the clinical phenomena of hormone independence and antiestrogen resistance in breast cancer.

We thank members of the Slingerland lab for helpful suggestions and critical review of the manuscript. This work was supported by grants from the U.S. Army Breast Cancer Research Program and the Burroughs Wellcome Fund to J.M.S., by an Ontario Graduate Studies Award to J.C.H.D., and by Medical Research Council funding to A.M.

- Musgrove, E. A. & Sutherland, R. L. (1994) *Cancer Biol.* **5**, 381–389.
- Henderson, B. E., Roos, R. & Bernstein, L. (1988) *Cancer Res.* **48**, 246–253.
- Jordan, V. C. (1995) *Breast Cancer Res. Treat.* **36**, 267–285.
- Early Breast Cancer Trialists' Collaborative Group (1998) *Lancet* **351**, 1451–1467.
- Sutherland, R. L., Green, M. D., Hall, R. E., Reddel, R. R. & Taylor, I. W. (1983) *Eur. J. Cancer Clin. Oncol.* **19**, 615–621.
- Osborne, C. K., Boldt, D. H., Clark, G. M. & Trent, J. M. (1983) *Cancer Res.* **43**, 3583–3585.
- Watts, C. K. W., Brady, A., Sarcevic, B., deFazio, A. & Sutherland, R. L. (1996) *Mol. Endocrinol.* **9**, 1804–1813.
- Nicholson, R. I., Francis, A. B., McClelland, R. A., Manning, D. L. & Gee, J. M. W. (1994) *Endocr. Relat. Cancer* **3**, 1–13.
- Encarnacion, C. A., Cioanca, D. R., McGuire, W. L., Clark, G. M., Fuqua, S. A. & Osborne, C. K. (1993) *Breast Cancer Res. Treat.* **26**, 237–246.
- Robertson, J. F. R. (1996) *Br. J. Cancer* **73**, 5–12.
- Howell, A., DeFriend, D., Robertson, J., Blamey, R. & Walton, P. (1995) *Lancet* **345**, 29–30.
- Howell, A., DeFriend, D. J., Robertson, J. F. R., Blamey, R. W., Anderson, L., Anderson, E., Sutcliffe, F. A. & Walton, P. (1996) *Br. J. Cancer* **74**, 300–308.
- Perlmann, T. & Evans, R. M. (1997) *Cell* **90**, 391–397.
- Beato, M., Chavez, S. & Truss, M. (1996) *Steroids* **61**, 240–251.
- Katzenellenbogen, J. A., O'Malley, B. W. & Katzenellenbogen, B. S. (1996) *Mol. Endocrinol.* **10**, 119–131.
- Migliaccio, A., DiDomenico, M., Castona, C., DeFalco, A., Bontempo, P., Nola, E. & Auricchio, F. (1996) *EMBO J.* **15**, 1292–1300.
- Collins, P. & Webb, C. (1999) *Nat. Med.* **5**, 1130–1131.
- van der Kwast, T. H., Schalken, J., Ruizeveld de Winter, J. A., van Vroonhoven, C. C., Mulder, E., Boersma, W. & Trapman, J. (1991) *Int. J. Cancer* **48**, 189–193.
- Ruizeveld de Winter, J. A., Janssen, P. J., Sleddens, H. M., Verleun-Mooijman, M. C., Trapman, J., Brinkmann, A. O., Santerse, A. B., Schroder, F. H. & van der Kwast, T. H. (1994) *Am. J. Pathol.* **144**, 735–746.
- Solomon, M. J. & Kaldis, P. (1998) *Results Probl. Cell Differ.* **22**, 79–109.
- Morgan, D. O. (1995) *Nature (London)* **374**, 131–134.
- Sherr, C. J. (1994) *Cell* **79**, 551–555.
- Reed, S. I., Bailly, E., Dulic, V., Hengst, L., Resnitzky, D. & Slingerland, J. (1994) *J. Cell Sci. Suppl.* **18**, 69–73.
- Sherr, C. J. & Roberts, J. M. (1999) *Genes Dev.* **13**, 1501–1512.
- Catzavelos, C., Bhattacharya, N., Ung, Y. C., Wilson, J. A., Roncari, L., Sandhu, C., Shaw, P., Yeager, H., Morava-Protzner, I., Kapusta, L., et al. (1997) *Nat. Med.* **3**, 227–230.
- Tan, P., Cady, B., Wanner, M., Worland, P., Cukor, B., Magi-Galluzzi, C., Lavin, P., Draetta, G., Pagano, M. & Loda, M. (1997) *Cancer Res.* **57**, 1259–1263.
- Wakasugi, E., Kobayashi, T., Tamaki, Y., Ito, Y., Miyashiro, I., Komoike, Y., Takeda, T., Shin, E., Takatsuka, Y., Kikkawa, N., et al. (1997) *Am. J. Clin. Pathol.* **107**, 684–691.
- Jiang, M., Shao, Z.-M., Wu, J., Lu, J.-S., Yu, L.-M., Yuan, J.-D., Han, Q.-X., Shen, Z.-Z. & Fontana, J. A. (1997) *Int. J. Cancer* **74**, 529–534.
- Tsilihas, J., Kapusta, L. & Slingerland, J. (1999) *Annu. Rev. Med.* **50**, 401–423.
- Saez, A., Sanchez, E., Sanchez-Beato, M., Cruz, M. A., Chacon, I., Munoz, E., Camacho, F. I., Martinez-Montero, J. C., Mollejo, M., Garcia, J. F., et al. (1999) *Br. J. Cancer* **80**, 1427–1434.
- Prall, O. W. J., Sarcevic, B., Musgrove, E. A., Watts, C. K. W. & Sutherland, R. L. (1997) *J. Biol. Chem.* **272**, 10882–10894.
- Planas-Silva, M. D. & Weinberg, R. A. (1997) *Mol. Cell. Biol.* **17**, 4059–4069.
- Foster, J. & Wimalasen, J. (1996) *Mol. Endocrinol.* **10**, 488–496.
- Soule, H. D., Vazquez, J., Long, A., Albert, S. & Brennan, S. (1973) *J. Natl. Cancer Inst.* **51**, 1409–1413.
- Petrocelli, T., Poon, R., Drucker, D., Slingerland, J. & Rosen, C. (1996) *Oncogene* **12**, 1387–1396.
- Sandhu, C., Garbe, J., Dakis, J., Pan, C.-H., Bhattacharya, N., Yaswen, P., Koh, J., Slingerland, J. & Stampfer, M. R. (1997) *Mol. Cell. Biol.* **17**, 2458–2467.
- LaBaer, J., Garrret, M., Steenson, M., Slingerland, J., Sandhu, C., Chou, H., Fattaey, A. & Harlow, H. (1997) *Genes Dev.* **11**, 847–862.
- Flanagan, W. M., Wolf, J. J., Olson, P., Grant, D., Lin, K. Y., Wagner, R. W. & Matteucci, M. D. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 3513–3518.
- St. Croix, B., Florenes, V., Rak, A., Flanagan, J. W., Bhattacharya, N., Slingerland, J. M. & Kerbel, R. S. (1996) *Nat. Med.* **2**, 1204–1210.
- Musgrove, E., Lilischkis, R., Cornish, A. L., Lee, S. L., Setlur, V., Seshari, R. & Sutherland, R. L. (1995) *Int. J. Cancer* **63**, 584–591.
- Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M. & Sherr, C. J. (1999) *EMBO J.* **18**, 1571–1583.
- Watts, C. K., Sweeney, K. J. E., Warlters, A., Musgrove, E. A. & Sutherland, R. L. (1994) *Breast Cancer Res. Treat.* **31**, 95–105.
- Prall, O. W. J., Rogan, E. M., Musgrove, E. A., Watts, C. K. W. & Sutherland, R. L. (1998) *Mol. Cell. Biol.* **18**, 4499–4508.
- Sheaff, R. J., Singer, J. D., Swanger, J., Smitherman, M., Roberts, M. J. & Clurman, B. E. (2000) *Mol. Cell* **5**, 403–410.
- Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M. & Clurman, B. E. (1997) *Genes Dev.* **11**, 1464–1478.
- Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A. & Pagano, M. (1999) *Genes Dev.* **13**, 1181–1189.
- Vlach, J., Hennecke, S. & Amati, B. (1997) *EMBO J.* **16**, 5334–5344.
- Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H. & Okayama, H. (1994) *EMBO J.* **13**, 1549–1556.
- Hoffmann, I., Draetta, G. & Karsenti, E. (1994) *EMBO J.* **13**, 4302–4310.
- Rivard, N., L'Allemain, G., Bartek, J. & Pouyssegur, J. (1996) *J. Biol. Chem.* **271**, 18337–18341.
- Coats, S., Flanagan, M., Nourse, J. & Roberts, J. M. (1996) *Science* **272**, 877–880.
- Coats, S., White, P., Fero, M. L., Lacy, S., Chung, G., Randel, E., Firpo, E. & Roberts, J. M. (1999) *Curr. Biol.* **9**, 163–173.
- Bates, S., Parry, D., Bonetta, L., Vousden, K., Dickson, C. & Peters, G. (1994) *Oncogene* **9**, 1633–1640.
- Parry, D., Bates, S., Mann, D. J. & Peters, G. (1995) *EMBO J.* **14**, 503–511.
- Matsushima, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. A. & Kato, J.-Y. (1994) *Mol. Cell. Biol.* **14**, 2066–2076.
- Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F. & Rolfe, M. (1995) *Science* **269**, 682–685.
- Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H. & Zhang, H. (1999) *Curr. Biol.* **9**, 661–664.
- Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U. & Krek, W. (1999) *Nat. Cell Biol.* **1**, 207–214.
- Carrano, A., Eytan, E., Hershko, A. & Pagano, M. (1999) *Nat. Cell Biol.* **1**, 193–199.
- Alessandrini, A., Chiaur, D. S., Erikson, R. & Pagano, M. (1997) *Leukemia* **11**, 342–345.
- Kawada, M., Yamagoe, S., Murakami, Y., Suzuki, K., Mizuno, S. & Uehara, Y. (1997) *Oncogene* **15**, 629–637.